

This Page Is Inserted by IFW Operations
and is not a part of the Official Record

BEST AVAILABLE IMAGES

Defective images within this document are accurate representations of the original documents submitted by the applicant.

Defects in the images may include (but are not limited to):

- BLACK BORDERS
- TEXT CUT OFF AT TOP, BOTTOM OR SIDES
- FADED TEXT
- ILLEGIBLE TEXT
- SKEWED/SLANTED IMAGES
- COLORED PHOTOS
- BLACK OR VERY BLACK AND WHITE DARK PHOTOS
- GRAY SCALE DOCUMENTS

IMAGES ARE BEST AVAILABLE COPY.

**As rescanning documents *will not* correct images,
please do not report the images to the
Image Problem Mailbox.**



(11) **EP 1 213 354 A2**

(12) **EUROPEAN PATENT APPLICATION**

(43) Date of publication:
12.06.2002 Bulletin 2002/24

(51) Int Cl.7: **C12N 15/53, C12N 9/04,
C12N 1/21, C12P 7/42,
C12P 7/62**

(21) Application number: **01310251.2**

(22) Date of filing: **07.12.2001**

(84) Designated Contracting States:
**AT BE CH CY DE DK ES FI FR GB GR IE IT LI LU
MC NL PT SE TR**
Designated Extension States:
AL LT LV MK RO SI

(30) Priority: **07.12.2000 JP 2000372704
15.01.2001 JP 2001006144
02.02.2001 JP 2001026594
11.06.2001 JP 2001175175**

(83) Declaration under Rule 28(4) EPC (expert
solution)

(71) Applicant: **Sumitomo Chemical Company,
Limited
Chuo-ku Osaka 541-8550 (JP)**

(72) Inventors:
• **Asako, Hiroyuki
Minoo-shi, Osaka (JP)**
• **Matsumura, Kenji
Minoo-shi, Osaka (JP)**
• **Shimizu, Masatoshi
Toyonaka-shi, Osaka (JP)**
• **Ito, Nobuya
Tayama-shi, Toyama (JP)**
• **Wakita, Ryuhei
Toyonaka-shi, Osaka (JP)**

(74) Representative: **Cresswell, Thomas Anthony
J.A. KEMP & CO.
14 South Square
Gray's Inn
London WC1R 5JJ (GB)**

(54) **Process for producing optically active 4-halo-3-hydroxybutanoate**

(57) There are provided a polynucleotide sequence coding for an amino acid sequence capable of preferentially producing (S)-4-bromo-3-hydroxybutanoate by asymmetrically reducing 4-bromo-3-oxobutanoate, a DNA construct having a promoter in operative linkage

with the polynucleotide sequence, a recombinant vector comprising the polynucleotide sequence, a transformant, a recombinant vector and the like.

EP 1 213 354 A2

Description

Background of the Invention

[0001] Optically active 4-halo-3-hydroxy butanoate and related compounds are known as a useful intermediate compounds for the production of pharmaceuticals and agrochemicals.

[0002] Various methods for optically active 4-halo-3-hydroxybutanoate and compounds derivatized therefrom have been known so far. however, these methods are not always satisfactory in yield or industrial applicability.

Summary of the invention

[0003] According to the present invention, optically active 4-halo-3-hydroxy butanoate and related compounds can be readily obtained in an industrially desirable manner.

[0004] The present invention provides:

1. a polynucleotide sequence having:

- a) a polynucleotide sequence coding for an amino acid sequence of SEQ ID NO: 1;
- b) a polynucleotide sequence that hybridizes, under stringent conditions, with a polynucleotide sequence coding for an amino acid sequence of SEQ ID NO: 1, the amino acid sequence being an amino acid sequence of a protein capable of preferentially producing (S)-4-bromo-3-hydroxybutanoate by asymmetrically reducing 4-bromo-3-oxobutanoate; or
- c) a polynucleotide sequence of SEQ ID NO: 2 (hereinafter referred to as "the present gene or the present polynucleotide").

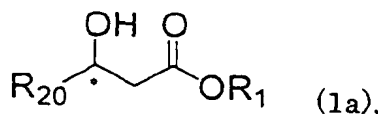
2. a protein having:

- i) an amino acid sequence of SEQ ID NO: 1;
- ii) an amino acid sequence encoded by a polynucleotide sequence that hybridizes under stringent conditions with a polynucleotide sequence of SEQ ID NO: 2 coding for an amino acid sequence of a protein capable of preferentially producing (S)-4-bromo-3-hydroxybutanoate by asymmetrically reducing 4-bromo-3-oxobutanoate; or
- iii) an amino acid sequence of SEQ ID NO: 1, wherein one or more amino acids are deleted, replaced or added, said amino acid sequence being an amino acid sequence of a protein capable of preferentially producing (S)-4-bromo-3-hydroxybutanoate by asymmetrically reducing 4-bromo-3-oxobutanoate (hereinafter referred to as "the present protein or enzyme");

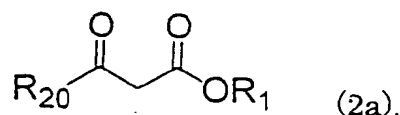
3. a process for producing (S)-4-halo-3-hydroxybutanoate, which comprises reacting 4-halo-3-oxobutanoic acid ester with the protein as defined above, a transformant, which produces the protein or a treated product thereof

4. a process for producing an optically active

3-hydroxybutanoate of formula (1a):



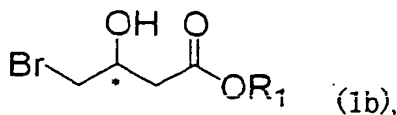
wherein R_1 represents an alkyl group, and R_{20} represents a methyl group which may be substituted with a halogen atom, which process comprises reacting 3-oxobutanoate of formula (2a):



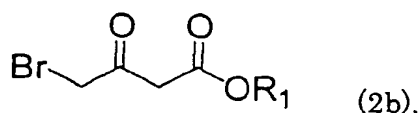
wherein R_1 and R_{20} represent the same as defined above, with a whole cells of a microorganism or a treated

product thereof, which microorganism belongs to *Penicillium citrinum*, *Cryptococcus humicolus*, or *Bacillus alvei* and is capable of asymmetrically reducing the oxo group at 3-position of the compound of formula (2a) to corresponding 3-hydroxy group;

5. a process for producing an optically active 4-bromo-3-hydroxybutanoate of formula (1b):



wherein R_1 represents a (C2-C8)alkyl group, which process comprises reacting 4-bromo-3-oxobutanoate of formula (2b):



wherein R_1 represents the same as defined above, with an enzyme having:

iv) an amino acid sequence of SEQ ID NO: 34;

v) an amino acid sequence encoded by a polynucleotide sequence that hybridizes, under stringent conditions, with a polynucleotide sequence of SEQ ID NO: 35, wherein the amino acid sequence is an amino acid sequence of a protein capable of preferentially producing optically active 4-bromo-3-hydroxybutanoate by asymmetrically reducing 4-bromo-3-oxobutanoate; and

vi) an amino acid sequence of SEQ ID NO: 34, wherein one or more amino acids are deleted, replaced or added, said amino acid sequence being an amino acid sequence of a protein capable of preferentially producing optically active 4-bromo-3-hydroxybutanoate by asymmetrically reducing 4-bromo-3-oxobutanoate (hereinafter referred to as "the instant enzyme"); and

6. A process for producing 4-cyano-3-hydroxybutanoic acid, which comprises reacting 4-bromo-3-hydroxybutanoic acid ester with a metal cyanide in the presence of an alkaline earth metal hydroxide and an alkaline earth metal halogenide.

Detailed Description of the Invention

[0005] First, a description will be made to the first aspect of the present invention drawn to the present gene. The present gene may be a wild-type gene or an artificially produced gene by introducing a mutation through site-specific mutation introduction method and/or a mutagenic treatment as described below into the wild type-gene. The wild-type gene can be found out and obtained among microorganisms capable of preferentially producing (S)-4-bromo-3-hydroxybutanoate ester by asymmetrically reducing 4-bromo-3-oxo-butanoate ester. Examples of the microorganism include, for example, microorganisms belonging to the genus *Penicillium* such as *Penicillium citrinum*.

[0006] Examples of the present gene include, for example, polynucleotides shown above in a) to c) and the like.

[0007] The stringent conditions in b) above include conditions that are referred to in a Southern hybridization method as described, for example, in the "Cloning and Sequence" (supervised by K. Watanabe, edited by M. Sugiyama and published from Noson Bunkasha Co., Ltd. in 1989). More specifically, examples of the polynucleotide include, for example, a polynucleotide that is defined by the following conditions: (1) the polynucleotide hybridizes with a polynucleotide coding for the amino acid sequence of SEQ ID NO: 1 to form a hybrid with the polynucleotide coding for the amino acid sequence of SEQ ID NO: 1 under high ion concentration conditions (e.g., 6XSSC (900mM sodium chloride, 90mM sodium citrate) at 65°C, and (2) the polynucleotide hybrid formed is maintained as a hybrid after being kept at 65°C for 30 minutes under a low ion concentration (e.g. 6XSSC (15mM sodium chloride and 1.5mM of sodium citrate)).

[0008] Examples of the present gene also includes, for example,

(d) a polynucleotide coding for an amino acid sequence of SEQ ID NO: 1 in which a part of the nucleotides is deleted, substituted or added; and

(e) a polynucleotide sequence having 80% or more sequence identity with the polynucleotide sequence coding for an amino acid sequence of SEQ ID NO: 1; and the like.

[0009] These polynucleotide sequences may be a polynucleotide obtained by cloning from the wild-type gene or may be a polynucleotide obtained from the cloned gene or a recombinant gene therefrom by artificially introducing partial deletion, substitution or addition of nucleotide(s) in the polynucleotide sequence coding for the amino acid sequence of SEQ ID NO: 1. Specific examples thereof include, for example, a polynucleotide sequence of SEQ ID NO: 2, and a polynucleotide sequence of SEQ ID NO: 28, and the like.

[0010] The polynucleotides a) to d) as defined above of the present invention can be prepared, for example, by preparing a cDNA library and conducting PCR (polymerase chain reaction) using the cDNA as a template and a suitable primer. The cDNA library can be prepared, for example, from microorganisms belonging to the genus *Penicillium* such as *Penicillium citrinum* according to genetic engineering methods (e.g. "A new cell engineering experiment protocol" (edited by the Cancer Research Unit, Medical Science Research Laboratory, the University of Tokyo, Shujinsha, Co., Ltd., 1993).

[0011] The polynucleotide sequence of SEQ ID NO: 28 can be amplified by carrying out PCR using the aforementioned cDNA library as a template and using an oligonucleotide sequence of SEQ ID NO: 23 and an oligonucleotide sequence of SEQ ID NO: 24 as primers.

[0012] Examples of the PCR conditions include, for example, a reaction condition in which a reaction mixture containing four types of dNTP, each of which are added so that the final concentration thereof in the mixture is 20 μ M, 15 pmol of two types of oligonucleotide as primers, 1.3 U of Taq polymerase and the cDNA library as a template is heated at 97 °C (for 2 minutes), 10 times of a cycle of reacting at 97 °C (for 0.25 minutes), 50 °C (for 0.5 minutes) and 72 °C (for 1.5 minutes), 20 times of a cycle of reacting at 97 °C (for 0.25 minutes), 55 °C (for 0.5 minutes) and 72 °C (for 2.5 minutes), and at 72 °C for 7 minutes.

[0013] A recognition sequence for a restriction enzyme may be added to the 5' terminal of the primer used for the PCR.

[0014] The present gene can also be prepared by PCR using the cDNA described above as a template and using, as primers, an oligonucleotide selected from a partial sequence of the polynucleotide sequence coding for the amino acid sequence of SEQ ID NO: 1 (e.g. an oligonucleotide comprising about 14 or more nucleotides at the 5' terminal of the polynucleotide sequence coding for the amino acid sequence of SEQ ID NO: 1) and an oligonucleotide comprising about 14 or more nucleotides complement to a nucleotide sequence at the vicinity of DNA inserting site of the vector used for constructing the cDNA.

[0015] Cloning of the amplified polynucleotide into a vector can provide a recombinant vector suitably used for the present invention according to methods as disclosed in "Molecular Cloning: A Laboratory Manual 2nd edition" (1989), Cold Spring Harbor Laboratory Press, "Current Protocols in Molecular Biology" (1987), John Wiley & Sons, Inc. ISBN 0-471-50338-X, etc. Examples of the vector that may be used include, for example, pUC119 (Takara Shuzo Co., Ltd.), pTV118N (Takara Shuzo Co., Ltd.), pBluescriptII (Toyobo Co., Ltd.), pCR2.1-TOPO™ (Invitrogen Co., Ltd.), pTrc99A (Pharmacia) and pKK223-3 (Pharmacia).

[0016] Alternatively, the present gene can be obtained by hybridizing a nucleotide sequence comprising about 15 or more nucleotides selected from the nucleotide sequence coding for the amino acid sequence of SEQ ID NO: 1, as a probe, with the cDNA inserted into a vector derived from a microorganism or a phage, thereby detecting the desired DNA which specifically binds to the probe, under a hybridization conditions described below.

[0017] Colony hybridization or plaque hybridization can be employed as a method for hybridizing a probe with a chromosomal DNA or cDNA library, and can be selected in accordance with the type of the vector used for preparation of the library.

[0018] Colony hybridization is preferably used together with the library prepared from a plasmid vector. For example, the library DNA is introduced into a host microorganism to produce a transformant, and the transformant is diluted, the diluted product is inoculated on an agar plate, and incubated until a colony appears.

[0019] Plaque hybridization is preferably used together with the library DNA prepared from a phage vector. For example, a host microorganism is mixed with the phage having the library, the resulting mixture is mixed with a soft cultivation agar medium under suitable conditions for infection, the mixture is then placed on an agar plate and incubated until formation of a plaque is confirmed.

[0020] A membrane is placed on the surfaces of the cultivation agar medium obtained by the hybridization methods as described above, thereby the transformant or the phage is adsorbed onto the membrane to give a transcribed membrane. After this membrane is subjected to alkaline treatment, it is neutralized and the DNA is then immobilized on the membrane. More specifically, in the case of plaque hybridization, nitro cellulose membrane or nylon membrane (e.g. Hybond-N⁺ (registered trademark of Amasham Inc.)) is placed on the aforementioned cultivation agar medium and is left standing for about one minute so that the phage particle is adsorbed by the membrane. Then, the membrane is immersed in alkali solution (e.g. 1.5 M sodium chloride and 0.5M sodium hydroxide) for about three minutes until the phage particle is dissolved. After the phage DNA is eluted onto the membrane, it is immersed in a neutralization

solution (e.g. 1.5 M sodium chloride and 0.5 M tris-hydrochloric acid buffer solution at pH 7.5) for about five minutes. Then, the membrane is washed with a solution (e.g. 0.3 M sodium chloride, 30 mM citric acid, 0.2 M tris-hydrochloric acid buffer solution at pH 7.5) for five minutes. Thereafter, it is heated at about 80 °C for about 90 minutes until the phage DNA is immobilized on the membrane.

[0021] Using the membrane prepared in the aforementioned steps, hybridization is performed with the DNA as a probe. Hybridization can be carried out according to the method disclosed, for example, in "Molecular Cloning: A Laboratory Manual 2nd edition (1989)" by J. Sambrook, E. F. Fritsch and T. Maniatis, Cold Spring Harbor Laboratory Press.

[0022] The DNA used as a probe can be labeled with either radioactive isotope or fluorophore. The probe DNA may be labeled, for example, by conducting PCR in which the dCTP in PCR reaction solution is replaced with (α -³²P) dCTP using the Random Primer Labeling Kit (Takara Shuzo Co., Ltd.) and the probe DNA is used as a template. The labeling of the probe DNA with the fluorophore can be accomplished, for example, by ECL Direct Nucleic Acid Labeling and Detection System produced by Amersham, and the like.

[0023] For example, hybridization can be carried out as follows:

[0024] The membrane prepared in the aforementioned step is immersed in a pre-hybridization solution containing sodium chloride at a concentration of 450 to 900 mM and sodium citrate at a concentration of 45 to 90 mM, sodium dodecyl sulfate (SDS) at a concentration of 0.1 to 1.0 wt%, denatured nonspecific DNA at a concentration of 0 to 200 μ l/ml, (a preferred pre-hybridization solution contains 900 mM sodium chloride, 90 mM sodium citrate, 1.0% SDS and 100 μ l/ml of modified calf-thymus DNA), and optionally albumin, Ficoll and polyvinyl pyrrolidone each at a concentration of 0 to 0.2 wt%, wherein the amount of the solution is 50 to 200 μ l per 1 cm² of the membrane, and maintained at 42 to 65 °C for one to four hours.

[0025] Then the membrane is immersed in a solution obtained by mixing the pre-hybridization solution as used above with the probe prepared in the aforementioned step in such an amount equivalent to 1.0 x 10⁴ to 2.0 x 10⁶ cpm per 1 cm² of membrane) and maintained at 42 to 65 °C for 12 to 20 hours, wherein the amount of the solution is 50 to 200 μ l per 1 cm² of the membrane.

[0026] After the hybridization, the membrane is taken out and is washed at 42 to 65 °C with a washing solution containing 15 to 300 mM sodium chloride, 1.5 to 30 mM sodium citrate and 0.1 to 1.0 wt% of SDS (a preferred, washing solution contains 15 mM sodium chloride and 1.5 mM sodium citrate and 1.0 % of SDS). The washed membrane is slightly rinsed with 2xSSC (300 mM sodium chloride 30 mM sodium citrate), and is then dried. This membrane is applied to the autoradiography, for example, to detect the position of the probe on the membrane, whereby position of the clone of the DNA hybrid is located on the original cultivating agar medium, and the clone containing the desired DNA can be picked up and isolated. The present gene can be obtained by cultivating the clone thus obtained.

[0027] The recombinant vector according to the present invention can be obtained by cloning of the DNA prepared in the aforementioned step into the vector according to the method disclosed in "Molecular Cloning: A Laboratory Manual 2nd edition (1989)", Cold Spring Harbor Laboratory Press, "Current Protocols in Molecular Biology"(1987), John Wiley & Sons, Inc. ISBN0-471-50338-X, etc. Examples of the vector include, for example, pUC119 (Takara Shuzo Co., Ltd.), pTV118N (Takara Shuzo Co., Ltd.), pBluescriptII (Toyobo Co., Ltd.), pCR2.1-TOPO™ (Invitrogen Co., Ltd.), pTrc99A (Pharmacia) and pKK223-3 (Pharmacia) and the like.

[0028] The aforementioned DNA sequence can be analyzed by the Dideoxy method described in the Proceeding of Natural Academy of Science, by F. Sanger, S. Nicklen and A. R. Coulson, U. S. A (1977) 74: PP. 5463-5467. In order to prepare samples for analysis of the DNA sequence, it is possible to use the commercially available reagent such as ABI PRISM Dye Terminator Cycle Sequencing Ready Reaction Kit of Parkin Elmer Inc. or the like.

[0029] The activity of the enzyme protein, encoded by the obtained DNA, produced by the transformant can be confirmed by introducing a vector having a promoter in operative linkage with the obtained DNA which locates downstream of the promoter as described below to a microorganism, and allowing the cultivated product of the transformant to react with 4-bromo-3-oxobutanoate to analyze the amount of (S)-4-bromo-3-hydroxybutanoate in the reaction.

[0030] The phrase "a promoter in operative linkage with the DNA" above means that the linkage with the promoter is made in such a way that the gene of the present invention will be expressed under the control of the promoter in a host cell introduced with the gene to transform the host cell. Examples of the promoter include, for example, the lactose operon promoter of *Escherichia coli*, tryptophan operon promoter of *Escherichia coli* and synthetic promoter such as tac promoter or trc promoter which can function in *Escherichia coli*. It is also possible to use the promoter controlling the expression of the present in *Penicillium citrinum* per se.

[0031] Generally, the present gene is introduced into the host cell in a form of a recombinant vector having a promoter in operative linkage with the DNA. A vector having a selective marker gene (e. g. antibiotic-resistance conferring gene such as kanamycin resistant gene and neomycin resistant gene), can also be used, whereby the transformant having the vector can be selected using the phenotyp of the relevant selective marker gene as an indicator.

[0032] Examples of the host cell into which the vector as described above or a DNA construct having a promoter in operative linkage with the present gene can be introduced include, for example, a microorganism belonging to *Escherichia*, *Bacillus*, *Corynebacterium*, *Staphylococcus*, *Streptomyces*, *Saccharomyces*, *Kluyveromyces* or *Aspergillus*.

[0033] A suitable method is selected to produce the transformant in accordance with the kind of cells used as the host. Examples of such methods include the calcium chloride method disclosed in "Molecular Cloning: A Laboratory Manual 2nd edition" (1989), Cold Spring Harbor Laboratory Press, and "Current Protocols in Molecular Biology" (1987), John Wiley & Sons, Inc. ISBN0-471-50338-X, etc., and the electroporation method described in "Method in Electro-

[0034] The present gene contained in the transformant can be confirmed by verifying the site of the restriction enzyme, analysis of the polynucleotide sequence, Southern hybridization, Western hybridization, etc., with respect to the DNA prepared subsequent to preparation of the vector DNA from the transformant, according to the normal method given in "Molecular Cloning: A Laboratory Manual 2nd edition" (1989), Cold Spring Harbor Laboratory Press.

[0035] The present protein will be explained below.

[0036] Examples of the amino acid sequence wherein one or more amino acids are deleted, substituted or added in the amino acid sequence of SEQ ID No: 1 include, for example, an amino acid sequence having additional 6-amino acid of Trp-Ile-Ser-Thr-Lys-Leu at the C-terminal of the amino acid sequence of SEQ ID NO: 1 in addition to those defined in i) to iii) above.

[0037] The present protein can be produced by cultivating the transformant containing the present gene.

[0038] Examples of the medium that may be used for cultivating the transfected host microorganisms include, for example, various mediums that contains a carbon source or a nitrogen source, an organic salt, an inorganic salt or the like in suitable amounts.

[0039] Examples of the carbon source include, for example, saccharides such as glucose, dextrin and sucrose, sugar alcohol such as glycerol, organic acid such as fumaric acid, citric acid and pyruvic acid, animal oil, plant oil and molasses. The amount of these carbon sources to be applied to the media is normally in the range of about 0.1 to 30 % (w/v) with respect to culture solution.

[0040] Examples of the nitrogen source include, for example, a naturally occurring organic nitrogen sources such as meat extract, peptone, yeast extract, malt extract, pulverized soy bean, Corn Steep Liquor, cottonseed, dried yeast and casamino acid; ammonium salt of inorganic acid such as amino acids and sodium nitrate, ammonium salt of inorganic acid such as ammonium chloride, ammonium sulfate and ammonium phosphate; ammonium salt of organic acid such as ammonium fumarate and ammonium citrate; and urea. Of these substances, an ammonium salt of organic acid, naturally occurring nitrogen source and amino acid can also be used as carbon sources in many cases. The amount of these nitrogen sources that may be applied to the media is normally in the range of about 0.1 to 30 % (w/v) with respect to culture medium.

[0041] Examples of the organic and inorganic salt include, for example, chloride, sulfate, acetate, carbonate and phosphate of calcium, sodium, magnesium, iron, manganese, cobalt and zinc. Specific examples thereof include, for example, sodium chloride, potassium chloride, magnesium sulfide, ferrous sulfide, manganese sulfide, cobalt chloride, zinc sulfate, copper sulfate, sodium acetate, calcium carbonate, monopotassium hydrogenphosphate, potassium dihydrogenphosphate and the like. The amount of these organic and/or inorganic salts that may be applied to the media is normally in the range of about 0.0001 to 5 % (w/v) with respect to culture medium.

[0042] A small amount of isopropyl thio- β -D-galactoside (IPTG) can be added, as an inducer of the production of the present protein, to the medium for cultivating the transformant having a promoter (e.g. tac promoter, trc promoter and lac promoter which are induced by allolactose) in operative linkage with the gene.

[0043] Cultivation of the transformant having the present gene can be conducted by a conventional method used for cultivating a host cell such as microorganism. For example, it can be done in the following method: liquid cultivation such as test tube shake cultivation, reciprocative shaking cultivation, jar fermenter cultivation and tank cultivation, and solid cultivation.

[0044] Cultivation temperature can be varied within the range where the microorganism can grow. It is normally within the range of 15 to 40 °C. Preferably, the pH value of the medium is set within the range from about 6 to 8. Cultivation time varies according to the cultivation conditions, and normally from about one to five days are preferred.

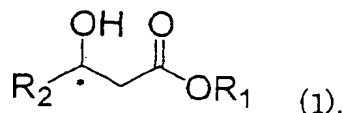
[0045] The present protein can be purified by a suitable purification method that may be usually applied to purify a protein. Examples thereof include following methods. Cells are collected by subjecting cultivated products of the transformant to centrifugation and collected cells are disrupted by physical disrupting such as ultrasonic treatment and French pressing, or chemical disrupting method using such a lytic enzyme as lysozyme. Impurities are removed from the obtained disrupted solution by centrifugation and membrane filter whereby cell-free extract is prepared. The extract is fractionated by a suitable separation and refining method such as cation exchange column chromatography, anion ion exchange column chromatography, hydrophobic column chromatography and gel column chromatography, whereby the present protein can be purified.

[0046] Examples of the support that may be used for the chromatography include, for example, a cellulose introduced with a carboxy methyl (CM) group, a diethyl amino ethyl (DEAE) group, a phenyl group or a butyl group, and a insoluble polymer support such as dextrin, agarose or the like. A commercially available columns charged with support can be used. Examples of the column available on the market include, for example, Q-Sepharose FF, Phenyl-Sepharose HP

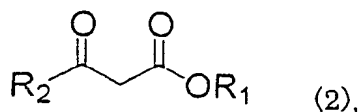
(trade names by Amasham Pharmacia Biotech) and TSK-gel G3000SW (trade name by Toso Co., Ltd.).

[0047] The fraction including the present protein can be selected, for example, by testing the capability of preferentially producing (S)-4-bromo-3-hydroxybutanoate by asymmetrically reducing 4-bromo-3-oxobutanoate.

[0048] Next, a description will be made to the method for producing (S)-4-halo-3-hydroxybutanoate of formula (1):



wherein R_1 represents an alkyl group, and R_2 represents a methyl group which is substituted with a halogen atom, which process comprises reacting 4-halo-3-oxobutanoate of formula (2):



wherein R_1 and R_2 represent the same as defined above with the present enzyme, a transformant which produces the enzyme, or a treated product thereof.

[0049] In the formula (1) and (2), R_1 denotes C1-C8 alkyl groups, and R_2 denotes methyl group substituted with a halogen atom or atoms.

[0050] Examples of the C1-C8 alkyl group represented by R_1 include methyl, ethyl, propyl, isopropyl, butyl, isobutyl, t-butyl, pentyl, hexyl, heptyl, and octyl groups.

[0051] Examples of a methyl group which is substituted with a halogen atom or atoms (e.g. fluorine, chlorine, bromine, or iodine) represented by R_2 include, for example, monofluoromethyl, monochloromethyl, monobromomethyl, difluoromethyl, dichloromethyl, dibromomethyl, trifluoromethyl, and trichloromethyl groups.

[0052] Specific-examples thereof include, for example, 4-halo-3-oxobutanoate ester include methyl 4-chloro-3-oxobutanoate, ethyl 4-chloro-3-oxobutanoate, propyl 4-chloro-3-oxobutanoate, methyl 4-bromo-3-oxobutanoate, ethyl 4-bromo-3-oxobutanoate, propyl 4-bromo-3-oxobutanoate, and octyl 4-bromo-3-oxobutanoate.

[0053] The production is usually carried out in the presence of water and reduced form nicotinamide adenine dinucleotide phosphate (hereinafter referred to as "NADPH"). Water used in this case may be an aqueous buffer solution. Examples of buffer agent that may be used for this aqueous buffer solution include, for example, an alkali phosphate metal salt such as sodium phosphate and potassium phosphate, alkali acetate metal salt such as sodium acetate aqueous solution and potassium acetate, and mixtures thereof.

[0054] The reaction may be conducted in the co-presence of an organic solvent. Examples of the organic solvent include, for example, an ether such as t-butyl methyl ether, diisopropyl ether, tetrahydrofuran or the like; an ester such as ethyl formate, ethyl acetate, propyl acetate, butyl acetate, ethyl propionate, butyl propionate or the like; a hydrocarbon such as toluene, hexane, cyclohexane, heptane, isooctane or the like; an alcohol such as methanol, ethanol, 2-propanol, butanol, t-butyl alcohol or the like; an organic sulfur compound such as dimethylsulfoxide or the like; a ketone such as acetone or the like; a nitrile such as acetonitrile or the like; and mixtures thereof.

[0055] The reacting of 4-halo-3-oxobutanoic acid ester of formula (2) with the present enzyme, a transformant which produces the enzyme, or a treated product thereof is usually conducted by stirring, shaking or mixing water, NADPH and 4-halo-3-oxobutanoate ester together with transformant or the treated substance, optionally in the presence of the organic solvent.

[0056] The pH of the reaction can be suitably set, normally within a range of 3 to 10. Reaction temperature is normally within the range of 0 to 60 °C in view of the stability of the 4-halo-3-oxobutanoate ester and a product thereof and reaction velocity.

[0057] Progress of the reaction can be monitored by, for example, tracing 4-halo-3-oxobutanoate ester in the reaction solution by means of high performance liquid chromatography or the like. The reaction time is usually in the range of 0.5 hour to 10 days.

[0058] After completion of the reaction, (S)-4-halo-3-hydroxybutanoate can be separated and recovered from the reaction mixture by a suitable methods. For example, the reaction mixture is subjected to post-treatment such as extraction, concentration of organic solvent, or a optional combination thereof, and the product may be further purified by column chromatography and distillation, if necessary.

[0059] The present protein, the transformant which produces the same or the treated product thereof can be used in various forms in the reaction. Examples of the applicable form thereof include, for example, the cultivated product of the transformant containing the present gene, the transformant cell containing the present gene; the treated product of thereof; cell-free extract; a crude purified protein; a purified protein; and the immobilized product thereof.

[0060] Examples of the treated product of the transformant include, for example, a freeze dried transformant, an organic solvent treated transformant, a dried transformant, a transformant ground by friction, a self-digested transformant, an ultrasonically treated transformant, an extract of the transformant and an alkali-treated transformant. Immobilized products can be obtained, for example, by the carrier bonding method (wherein the present protein is adsorbed onto an inorganic support such as silica gel, ceramic or the like, cellulose and ion exchange resin), and inclusion method (wherein the present protein is entrapped in the network structure of polymers such as polyacrylamide, a sulfur containing polysaccharide gel (e.g. carageenan gel), alginic acid gel and agar gel).

[0061] Sterilized transformants, as a treated product, is preferably used for an industrial production rather than the transformant containing the present gene per se, in view of less restriction imposed on production facilities.

[0062] Examples of the method for sterilizing the transformant for such a purpose include, for example, physical sterilization (heating, drying, freezing, light beams, ultrasound, filtration and application of electric current) and chemical sterilization (alkali, acid, halogen, oxidizing agents, sulfur, boron, arsenic, metal, alcohol, phenol, amine, sulfide, ether, aldehyde, ketone, cyan and antibiotics). Preferably selected is a method which accompanies lesser pollution or contamination in the reaction system and lesser loss of the enzymatic activity of the present protein.

[0063] The reaction is preferably conducted in the presence of NADPH. The NADPH is converted to the oxidized form β -nicotinic amide adenine dinucleotide phosphate (hereinafter referred to as "NADP⁺") with the progress of the reaction. Since the NADP⁺ can be converted back to the original NADPH by an enzyme capable of converting the NADP⁺ into NADPH, such an enzyme capable of converting the NADP⁺ into the NADPH may also be added to the reaction.

[0064] Examples of the enzyme capable of converting the NADP⁺ into the NADPH include, for example, a glucose dehydrogenase, an alcohol dehydrogenase, an aldehyde dehydrogenase, an amino acid dehydrogenase, an organic dehydrogenase (malic acid dehydrogenase) and the like.

[0065] The activity of the glucose dehydrogenase in the reaction system can be enhanced by adding glucose thereto.

[0066] The protein capable of converting the NADP⁺ into the NADPH can be applied or added to the reaction system in any form, for example, of the enzyme, a microorganism having the enzymatic activity or treated product thereof.

[0067] Alternatively, the microorganism having the activity described above may be a transformant having a gene coding for an enzyme capable of converting the NADP⁺ into the NADPH, or a treated product of the transformant. The treated product herein includes an equivalent thereof.

[0068] The transformant having gene of the enzyme capable of converting the NADP⁺ into the NADPH as described above and the present gene can be employed in this reaction.

[0069] Both of the genes may be introduced into a host cell by transfecting the same with a single vector having both of the genes, or with a plurality of recombinant vectors introduced with the respective gene. A further method of introducing the gene comprises introducing the present gene or both of the genes into chromosome of a host cell.

[0070] Examples of the method for introducing both of the genes into a single vector include, for example, a method for constructing a vector by linking expression control regions such as promoters and terminators to respective genes and a method of constructing a recombinant vector for expressing an operon containing multiple cistrons such as lactose operon.

[0071] Next a description will be made to the second aspect of the present invention relating to a process for producing an optically active 3-hydroxybutanoic acid ester of formula (1a) as defined above, which process comprises reacting 3-oxobutanoic acid ester of formula (2a) as defined above, with a whole cells of a microorganism or a treated product thereof, which microorganism belongs to *Penicillium citrinum*, *Cryptococcus humicola*, or *Bacillus alvei* and is capable of asymmetrically reducing the oxo group at 3-position of the compound of formula (2a) to corresponding 3-hydroxy group.

[0072] According to the second aspect of the present invention, the desired optically active ester compound can be readily produced in an industrially desirable manner.

[0073] Examples of a C1-C8 alkyl group represented by R₁ in the general formula (1) and (2) include methyl, ethyl, propyl, isopropyl, butyl, isobutyl, t-butyl, pentyl, hexyl, heptyl, and octyl groups.

[0074] Examples of a methyl group which may be substituted with a halogen atom or atoms represented by R₂₀ include methyl group and methyl groups substituted with a halogen atom or atoms. Specifically, examples of the methyl group substituted with a halogen atom or atoms include, for example, monofluoromethyl, monochloromethyl, monobromomethyl, difluoromethyl, dichloromethyl, dibromomethyl, trifluoromethyl, and trichloromethyl groups.

[0075] In the instant production method, the reaction of converting a 3-oxobutanoate of formula (1a) to an optically active 3-hydroxybutanoate of formula (2a) is achieved by employing cells or cell products of one or more microorganisms selected from the group consisting of *Penicillium citrinum*, *Cryptococcus humicola*, and *Bacillus alvei*.

[0076] Specific examples of cells or cell products of microorganisms which can be used in the reaction include those of *Penicillium citrinum* IFO 4631, *Cryptococcus humicolus* IFO 1527, and *Bacillus alvei* IFO 3343t.

[0077] The cultivation of microorganisms that can be used in this aspect of the invention can be conducted, for example, as described for the production of the transformant using the same cultivation medium and conditions in the first aspect of the present invention.

[0078] Microorganisms that may be used in the instant production method can be cultivated using media for various microorganisms comprising carbon and nitrogen sources, organic and inorganic salts, and others as appropriate.

[0079] Examples of carbon sources contained in the media include glucose, sucrose, glycerol, starch, organic acids, and molasses. Examples of nitrogen sources include yeast extract, meat extract, peptone, casamino acid, malt extract, soybean flour, corn steep liquor, cottonseed flour, dried yeast, ammonium sulfate, and sodium nitrate. Examples of organic and inorganic salts include sodium chloride, potassium chloride, sodium carbonate, monopotassium phosphate, dipotassium phosphate, calcium carbonate, ammonium acetate, manganese sulfate, copper sulfate, zinc sulfate, ferrous sulfate, and cobalt chloride.

[0080] Examples of methods for culturing include solid culture and liquid culture (such as tube culture, flask culture, and jar fermenter culture).

[0081] The temperature for cultivation and the pH of the medium are not limited to any specific values as long as they are within the range allowing the microorganisms to grow. The temperature for culturing, for example, can be in the range of 15 to 45 °C and the pH of the medium in the range of 4 to 8.

[0082] The cultivation period can be suitable set, depending on the cultivation conditions, and is usually one to seven days.

[0083] Microorganism cells obtained by cultivation can be used in the reaction according to the instant production methods without any treatment. For example, the cultivation liquid is utilized as it is, a method in which cells are harvested by centrifuging the cultivated liquid or the like, and wet cells obtained by washing the harvested cells with a buffer solution or water can be utilized.

[0084] Examples of products of microorganism cells which can be used in the present reaction include, for example, products made by subjecting cells obtained by cultivation to treatments with an organic solvent (acetone, ethanol, and the like), freeze-dried cells, alkali-treated cells, and products made by disrupting the cells physically or enzymatically. Furthermore, Examples of the cell products include those obtained by carrying out immobilization according to well-known procedures after the above-described treatments.

[0085] The reaction is usually carried out in the presence of water, and the water may be used in the form of buffer solutions. Examples of buffering agents that may be used in the buffer solutions include, for example, alkali metal salts of phosphoric acid such as sodium phosphates and potassium phosphates, and alkali metal salts of acetic acid such as sodium acetate and potassium acetate. In such cases, the pH of the aqueous layer during the reaction can be varied, if necessary, in the range where the reaction proceeds, and is usually in the range of 3 to 10.

[0086] The reaction can further utilize a hydrophobic organic solvent, such that the reaction can be carried out in the bilayer system of water and such a hydrophobic organic solvent. Examples of hydrophobic organic solvents that may be used in this case include, for example, an ester such as ethyl formate, ethyl acetate, propyl acetate, butyl acetate, ethyl propionate, butyl propionate or the like, an alcohol such as n-butyl alcohol, n-amyl alcohol, n-octyl alcohol or the like; an aromatic hydrocarbon such as benzene, toluene, xylene or the like, an ether such as diethyl ether, diisopropyl ether, methyl-t-butyl ether or the like, a halogenated hydrocarbon such as chloroform, 1,2-dichloroethane or the like, and a mixture thereof.

[0087] The concentration of the starting compound in the reaction, the 3-oxobutanoate of formula (1a), is usually 50 % (w/v) or less. In order that the concentration of the 3-oxobutanoate of formula (1a) in the reaction system be maintained at an almost constant level, the 3-oxobutanoate of formula (1a) may be added to the reaction system in a continuous or successive manner.

[0088] The reaction temperature is usually in the range of 0 to 60 °C.

[0089] In the reaction, saccharides such as glucose, sucrose, and fructose, alcohols such as ethanol, surfactants can optionally be added.

[0090] The reaction time is usually in the range of 0.5 hour to 10 days. The progress of the reaction can be monitored, for example, by determining the amount of the starting compound in the reaction solution with high performance liquid chromatography, gas chromatography, or the like, after the addition of a 3-oxobutanoate of formula (1a).

[0091] After completion of the reaction, an optically active 3-hydroxybutanoate of formula (2a) can be isolated by subjecting the reaction solution to usual post-treatments such as extraction with an organic solvent, concentration, etc. The isolated compound can be further purified with column chromatography, distillation, or the like, if necessary.

[0092] Next a description will be made to the third aspect of the present invention relating to a process for producing an optically active 4-bromo-3-hydroxybutanoate of formula (1b) as defined above, which process comprises reacting 4-bromo-3-oxobutanoate of formula (2b) as defined above with an enzyme having an amino acid sequence as defined in iv) to vi) above, which enzyme will be referred to as "the instant enzyme".

[0093] Examples of the C2-C8 alkyl group represented by R₁ in the 4-bromo-3-oxobutanoate of formula (1a) include, for example, ethyl, propyl, isopropyl, butyl, pentyl, hexyl, and octyl groups.

[0094] The polynucleotide sequence coding for an enzyme having the amino acid sequence shown in SEQ ID NO: 34 which can be employed in the instant production method is shown in SEQ ID NO: 35 (Appl. Microbiol. Biotechnol. (1999) 52, 386-392). The present polynucleotide may be naturally occurring or produced by a suitable mutation of the naturally occurring sequence such as point mutations, mutagenic treatments, or the like.

[0095] pUAR containing the DNA shown in SEQ ID NO: 35 is deposited under the Budapest Treaty with the Depositary, National Institute of Advanced Industrial Science and Technology.

[0096] The instant enzyme can be produced, for example, by cultivating microorganisms containing the instant polynucleotide of SEQ ID NO: 34.

[0097] The microorganism containing the instant polynucleotide of SEQ ID NO: 35 can be obtained by cultivating a transformed microorganism host cell transfected with a vector having the polynucleotide of SEQ ID NO: 35.

[0098] Examples of host cells include, for example, microorganisms belonging to *Escherichia*, *Bacillus*, *Corynebacterium*, *Staphylococcus*, *Streptomyces*, *Saccharomyces*, *Kluyveromyces*, and *Aspergillus*.

[0099] Introduction of the polynucleotide into the host cell can be accomplished by a suitable conventional method, depending on the kind of cells used as the host. Examples of the methods include calcium chloride methods described in "Molecular Cloning: A Laboratory Manual, 2nd edition" (1989), Cold Spring Harbor Laboratory Press, "Current Protocols in Molecular Biology" (1987), John Wiley & Sons, Inc., ISBN0-471-50338-X, etc., and electroporation methods described in "Methods in Electroporation: Gene Pulser/E. coli Pulser System", Bio-Rad Laboratories, (1993), etc.

[0100] Transformed microorganisms having the desired polynucleotide can be selected using, as an index, the phenotype or the like of a selectable marker gene contained in a vector, which include those described above in the first aspect of the present invention. It can be confirmed as to whether the transformed microorganisms carry the gene by preparing the vector DNA from the transformed microorganisms, and then subjecting the prepared vector DNA, for example, to a usual method (identification of restriction enzyme sites, analysis of the polynucleotide sequence, southern hybridization, or the like) as described in "Molecular Cloning" (J. Sambrook et al., Cold Spring Harbor, 1989) and others.

[0101] Cultivation of the transformed cells to express the desired enzyme can be conducted by using, as a medium for culturing the above-described microorganisms, various kinds of medium including those comprising carbon and nitrogen sources commonly used in culturing microorganisms, and organic and inorganic salts, and others as appropriate.

[0102] Examples of carbon sources include: saccharides such as glucose, dextrin and sucrose; sugar alcohols such as glycerol; organic acids such as fumaric, citric and pyruvic acids; animal and vegetable oils; and molasses. The amount of these carbon sources to be added into the medium may be usually in the range of 0.1 to 20 % (w/v) with respect to the entire medium volume.

[0103] Examples of nitrogen sources are organic or inorganic nitrogen sources, including: natural organic nitrogen sources or amino acids such as meat extract, peptone, yeast extract, malt extract, soybean flour, corn steep liquor, cottonseed flour, dried yeast and casamino acid; inorganic acid ammonium salts and nitrates such as sodium nitrate, ammonium chloride, ammonium sulfate and ammonium phosphate; organic acid ammonium salts such as ammonium fumarate and ammonium citrate; and urea. Among these, organic acid ammonium salts, natural organic nitrogen sources, amino acids, and the like can also be used as carbon sources in many cases. The amount of nitrogen sources to be added may be usually in the range of 0.1 to 30 % (w/v) with respect to the entire medium volume.

[0104] Examples of organic and inorganic salts may include chlorides, sulfates, acetates, carbonates and phosphates of potassium, sodium, magnesium, iron, manganese, cobalt, zinc, and others. Specifically, examples of such salts include sodium chloride, potassium chloride, magnesium sulfate, ferrous sulfate, manganese sulfate, cobalt chloride, zinc sulfate, copper sulfate, sodium acetate, calcium carbonate, sodium carbonate, monobasic potassium phosphate, dibasic potassium phosphate, and others. The amount of an organic or inorganic salt to be added may be usually in the range of 0.0001 to 5 % (w/v) with respect to the entire medium volume.

[0105] A small amount of isopropyl thio- β -D-galactoside (IPTG) can be added, as an inducer of the production of the instant protein, to the medium for cultivating the transformant having a promoter (e.g. tac promoter, trc promoter and lac promoter which are induced by allolactose) in operable linkage with the gene of the instant protein.

[0106] Cultivation can be carried out according to procedures commonly used for cultivating microorganisms. Examples of the procedures include, for example, liquid culture such as tube-shaking culture, reciprocally shaking culture, jar fermenter culture, and tank culture; and solid culture. When a jar fermenter is used, it is necessary to introduce sterile air into the jar fermenter, and an aeration rate of about 0.1 to about 2 times the volume of the cultivating medium per minute is usually chosen. The temperature for cultivation can be varied within the range where microorganisms stay viable, and usually, cultivation temperatures in the range of about 15 °C to about 40 °C are preferred. The pH of the medium is preferably in the range of about 6 to about 8. The period for culturing is varied depending on culturing conditions, and a period from about one day to about five days is desirable in usual cases.

[0107] Cells containing the instant enzyme thus produced, cell products, or purified materials of the instant enzyme can be used in this aspect of the invention.

[0108] Examples of cell products include, for example, freeze-dried cells, organic solvent treated cells, dried cells, triturated cells, cell autolysates, sonicated cells, cell extracts, alkali-treated cells, and in addition, products obtained by immobilizing these by commonly employed methods.

[0109] Purified enzyme can be produced, for example, by purifying the instant enzyme from cultures of microorganisms carrying the instant enzyme.

[0110] The instant enzyme isolated from cultures of the microorganisms, may be further purified a conventional method as used for the purification of ordinary proteins.

[0111] For example, cells are harvested from the cultivated microorganism by centrifugation or the like, and these cells are homogenized by physical disrupting procedures such as an ultrasonic treatment, Dynomill treatment and French press treatment, or by chemical disrupting procedures using surfactants or cell-lytic enzymes such as lysozyme. Insoluble materials are usually removed from the resultant homogenate by centrifugation, membrane filtration, or the like to prepare a cell-free extract, which will be then fractionated through separation and purification procedures such as cation exchange column chromatography, anion exchange column chromatography, hydrophobic column chromatography, gel column chromatography, and the like to purify the instant enzyme. Examples of the support that may be used for the chromatography include, for example, resin support such as cellulose, dextran and agarose, having an carboxymethyl (CM) DEAE, phenyl, butyl group, or the like. Commercially available carrier-packed columns may also be used such as O-Sepharose FF, Phenyl-Sepharose HP (trade name, both manufactured by Amersham Pharmacia Biotech), TSK-gel G3000SW (trade name, manufactured by Toso Co., Ltd.), and others.

[0112] Next the instant production method will be described.

[0113] 4-bromo-3-oxobutanoate of formula (1b) is converted to an optically active 4-bromo-3-hydroxybutanoate of formula (2b) by subjecting a 4-bromoacetoacetic acid ester compound of formula (1b) to the reaction with the instant enzyme.

[0114] The reaction is usually carried out in the presence of water, and the water can be in the form of buffer solutions. Examples of buffering agents that may be used include, for example, alkali metal phosphates such as sodium phosphates and potassium phosphates; alkali metal salts of acetic acid such as sodium acetate and potassium acetate.

[0115] The pH in this case can be varied within the range where the reaction proceeds. The pH is usually optionally set within the range of pH 4 to 10.

[0116] The amount of the buffer that may be used is usually 100 parts by weight or less per one part by weight of a 4-bromo-3-oxobutanoate of formula (1b).

[0117] The reaction temperature for the above-described reaction is between 0 and 70 °C in view of stability and reaction rate of the instant enzyme, and preferably between 10 and 40 °C.

[0118] The reaction can also be carried out in the co-presence of an organic solvent. Examples of the organic solvent include, for example, an ether such as tetrahydrofuran, t-butyl methyl ether, isopropyl ether or the like, a hydrocarbon such as toluene, hexane, cyclohexane, heptane, isooctane, decane or the like, an alcohol such as t-butanol, methanol, ethanol, isopropanol, n-butanol or the like, a sulfoxide such as dimethylsulfoxide or the like; a ketone such as acetone or the like; a nitrile such as acetonitrile or the like, and a mixture thereof.

[0119] The amount of an organic solvent that may be used in the reaction is usually 100 parts by weight or less, and preferably 50 parts by weight or less, per one part by weight of 4-bromoacetoate of formula (1b).

[0120] A coenzyme (e.g., NADH, NADPH) may also be added for the above-described reaction. The amount of the coenzyme that may be used in the reaction is usually 0.5 part or less, and preferably 0.1 part or less, per one part by weight of 4-bromo-3-oxobutanoate of formula (1b), by weight.

[0121] Additional substances described below is preferably added together with the coenzyme in order to enhance the coenzyme efficiency:

1) compound(s) such as formic acid, glucose, isopropanol, 2-butanol, 2-pentanol, 2-hexanol, 2-heptanol, 2-octanol, and the like,

wherein the amount of these compounds the may be used is 100 parts or less by weight, and preferably 10 parts or less by weight, per one part by weight of 4-bromo-3-oxobutanoate of formula (1b); and

2) dehydrogenase such as formate dehydrogenase, glucose dehydrogenase, and the like, wherein the amount of dehydrogenase to be used is 0.1 part or less by weight, and preferably 0.05 part or less by weight, per one part by weight of 4-bromo-3-oxobutanoate of formula (1b).

[0122] The reaction can be carried out, for example, by stirring and shaking a mixture of water, a 4-bromo-3-oxobutanoate of formula (1b), the instant enzyme, a coenzyme, if necessary, an organic solvent, and others.

[0123] The progress of the reaction can be monitored, for example, by checking the amount of the starting compound in the reaction solution with high performance liquid chromatography, gas chromatography, or the like. The range of

the reaction time is usually a period varying from five minutes to four days.

[0124] After completion of the reaction, for example, the reaction solution can be extracted with an organic solvent such as hexane, heptane, tert-butyl methyl ether, ethyl acetate, toluene, or the like, and the organic layer can be dried and concentrated, thereby obtaining the desired product. The product can be further purified by column chromatography or the like, if necessary.

[0125] The instant production method can also be performed, for example, by using a polynucleotide constructed by subjecting the polynucleotide of SEQ ID NO: 35, for example, to well-known techniques for causing a point mutation or mutations in the polynucleotide such as the site-specific mutation, a recombinant method by cleaving the polynucleotide selectively or deleting, substituting or adding a suitably selected nucleotide or nucleotides, and ligating the resulting polynucleotide sequences to obtain a desirably modified polynucleotide, or an oligonucleotide mutation method, thereby producing a polynucleotide that codes for an enzyme capable of reducing a 4-bromo-3-oxobutanoate of formula (1b) to an optically active 4-bromo-3-hydroxybutanoate of formula (2b); and carrying out the preparation of transformants, cultivation and reaction in the above described method.

[0126] Next a description will be made to the fourth aspect of the present invention relating to a process for producing 4-cyano-3-hydroxybutanoic acid, which comprises reacting 4-bromo-3-hydroxybutanoic acid ester with a metal cyanide in the presence of an alkaline earth metal hydroxide and an alkaline earth metal halogenide.

[0127] According to this aspect of the present invention, 4-cyano-3-hydroxybutanoic acid or ester thereof can be obtained in a good yield.

[0128] A racemic or optically active 4-bromo-3-hydroxybutanoic acid ester can be used in this process. Examples of the ester include, for example, a lower alkyl ester of 4-bromo-3-hydroxybutanoic acid and optically active isomer thereof. Specific examples of the lower alkyl ester include, for example, C1-C5 alkyl esters such as a methyl, ethyl, propyl, isopropyl, or butyl ester.

[0129] Examples of the metal cyanide to be used include, for example, sodium cyanide and potassium cyanide.

[0130] Examples of the alkaline earth metal hydroxide used include, for example, magnesium hydroxide, calcium hydroxide, and barium hydroxide.

[0131] Examples of the alkaline earth metal halide used include, for example, alkaline earth metal chloride and alkaline earth metal bromide, and preferred is calcium chloride.

[0132] The reaction of the 4-bromo-3-hydroxybutanoic ester and metal cyanide in the presence of an alkaline earth metal hydroxide and an alkaline earth metal halide is preferably carried out in the presence of water. An organic solvent can also be added, if necessary.

[0133] The reaction temperature is normally from -10 to 100 °C, or preferably, from -10 to 40 °C.

[0134] An amount of the metal cyanide that may be used in the reaction is normally from 0.8 to 2 moles per mol of 4-bromo-3-hydroxybutanoic acid ester.

[0135] An amount of the alkaline earth metal hydroxide and alkaline earth metal halide to be used in the reaction is preferably from 0.8 to 5 moles per mol of 4-bromo-3-hydroxybutanoic acid ester.

[0136] The progress of the reaction can be monitored by checking the amount of 4-bromo-3-hydroxybutanoic acid using such an analysis means as the gas chromatography, the high-performance liquid chromatography, the thin layer chromatography or the like.

[0137] After completion of the reaction, for example, an inorganic acid such as hydrochloric acid is usually added to the reaction solution, thereafter the resulting mixture may be extracted with an organic solvent and the obtained organic layer is typically concentrated to obtain the 4-cyano-3-hydroxybutanoic acid.

[0138] The 4-Bromo-3-hydroxybutanoic ester and metal cyanide are made to react in the presence of an alkaline earth metal hydroxide and an alkaline earth metal halide, and the resulting mixture is then allowed to react with dialkyl sulfuric acid to produce the 4-cyano-3-hydroxybutanoic acid ester.

[0139] The reaction of the 4-cyano-3-hydroxybutanoic acid obtained as above with a dialkyl sulfuric acid is usually carried out in the presence of a base, preferably in a solvent.

[0140] Examples of the solvent that may be used in the reaction include, for example, an ester such as ethyl acetate, butyl acetate or the like, an ether such as tetrahydrofuran, 1,4-dioxane or the like, water and a mixture thereof. Examples of the base include, for example, tertiary amines such as pyridine, 4-dimethylamino pyridine, triethylamine and diisopropylethylamine.

[0141] The reaction is conducted normally from 0 to 100 °C.

[0142] The dialkyl sulfuric acid is usually used in an amount of from 1 to 2 moles and the base is usually used in an amount of from 1 to 3 moles per mol of the 4-cyano-3-hydroxybutanoic acid.

[0143] The progress of the reaction can be monitored by checking the amount of the 4-bromo-3-hydroxybutanoic acid using analytical means such as gas chromatography, the high-performance liquid chromatography, thin layer chromatography or the like.

[0144] After completion of the reaction, for example, saturated sodium bicarbonate solution is added to the reaction mixture, thereafter, the mixture is usually extracted with an organic solvent and the obtained organic layer is concen-

trated to give the 4-cyano-3-hydroxybutanoic acid ester.

[0145] The reaction of the dialkyl sulfate with the resulting 4-cyano-3-hydroxybutanoic acid is usually carried out after isolating the acid or the reaction can be conducted by reacting the dialkyl sulfate with the reaction mixture containing 4-cyano-3-hydroxybutanoic acid produced, wherein dialkyl sulfuric acid is preferably added to the reaction mixture.

[0146] The following describes the present invention in detail with reference to embodiments, which by no means limit the scope of the present invention.

Reference Example 1

[0147] 100ml of medium (potato dextrose broth (by Vecton Dickinson) dissolved to water at a ratio of 24 g/L) was put into a 500 ml flask, and was sterilized at 121 °C for 15 minutes. Then 0.5 ml of one strain of *Penicillium citrinum* IF04631 cultivated in the medium of the same composition (cultured by shaking at 30 °C for 48 hours) was added to it, and was shaken and cultured at 30 °C for 72 hours. Then the obtained cultivation solution was centrifuged (8000 xg for ten minutes), and the resulted precipitation was collected. This precipitation was washed in 50 ml of 20 mM potassium phosphate buffer (pH7) three times to obtain about 1.0 g of wet bacterial cell.

[0148] 10 g of said wet bacterial cell was used to prepare total RNA according to the guanidine phenol chloroform thiocyanate method, and about 1.5 mg of total RNA was obtained. Further, about 9.3 µg of RNA containing poly(A) was obtained from 0.5mg of total RNA using oligotex(dT)30-Super (Takara Shuzo Co., Ltd.).

[0149] cDNA library was prepared according to the Gubler and Hoffman method as follows. A single-stranded cDNA was prepared using the RNA (3.0 µg) containing the above poly (A), Oligo(dT) 18-linker primer ((including XhoI site) Takara Shuzo Co., Ltd.) and RAV-2 Rtae and SuperScriptII Rtae. *E. Coli* DNA polymerase, *E.coli* Rnae/*E. coli* DNA ligase mixture and T4 DNA polymerase was added to this reaction solution to carry out double stranded cDNA synthesis and smooth terminalization. This was followed by ligation between this double stranded cDNA and EcoRI-NotI-BamHI adaptor (Takara Shuzo Co., Ltd.). The DNA was ligated, phosphorylated and was digested by XhoI. The low molecular weight DNA was removed by the spin column (Takara Shuzo Co., Ltd.), and λ ZapII (EcoRI-XhoI digest) and ligation were performed. After that, packaging was made using the *in vitro* packaging kit (Stratagene Inc.), whereby a cDNA library (hereinafter referred to as "cDNA library" (A)) was obtained.

Example 1

[0150] (1) 23 g of wet bacterial cells of the *Penicillium citrinum* IF04631 strain which were prepared under the same conditions as those of the Reference Example 1 were suspended in 50 mM potassium phosphate buffer (pH 7.0) and were disrupted by Dynomill (manufactured by Symal Enterprise Inc., a glass bead 0.1 to 0.2 mm in diameter, at 3000 rpm for 30 minutes). The disrupted solution obtained in this step was subjected to centrifugal separation (10,000 xg for 10 minutes). The supernatant was further subjected to ultracentrifugation (100,000 xg for 120 minutes) to give 160 ml of untracentrifuged supernatant.

[0151] Ammonium sulfate was gradually added to 160 ml of the ultracentrifuged supernatant obtained in the aforementioned step until the concentration reached 1.5M. Then the solution is spread over hydrophobic interaction chromatography column [Hi-LoadPhenyl (26/10) (manufactured by Amasham Pharmacia Biotech), which was equilibrated with BIS-TRIS-PROPANE buffer (20 mM, pH 7.0) containing 1.5M ammonium sulfate], and eluted, as a mobile phase, with a BIS-TRIS-PROPANE buffer (concentration gradient of ammonium sulfate: 1.5M → 0.6M) containing ammonium sulfate. Thus, 20 ml of eluted fraction with an ammonium sulfate concentration of 1.1 to 0.9M was obtained as a fraction having a reducing enzyme activity.

[0152] The eluted fraction was subjected to desalination and was replaced by Tris-HCl buffer solution (20mM, pH 7.7), and then spread over the ion exchange chromatography column [Hi-Load Q Sepharose (16/10) (manufactured by Amasham Pharmacia Biotech), which is buffered by Tris-HCl buffer solution (20mM, pH7.7)]. Tris-HCl buffer solution (concentration gradient of sodium chloride: 0 → 0.5M) containing sodium chloride was used as a mobile phase to elute. Thus, 3 ml of a fraction containing a sodium chloride at a concentration of 0.02 to 0.08M was obtained as a fraction having reducing enzyme activity. The fraction was concentrated and the concentrated solution was subjected to gel filtration (column: Superdex 200 (10/30) (manufactured by Amasham Pharmacia Biotech)) [mobile phase: BIS-TRIS-PROPANE buffer (20mM, pH7.0)]. Thus, 1 ml of a fraction corresponding to a molecular weight of about 33000 Dalton (hereinafter referred to as "active fraction (A)") was obtained as a fraction having a reducing enzyme activity.

[0153] The reducing enzyme activity of the fraction obtained by chromatography or the like was measured according to the following steps.

[0154] The eluted fraction obtained by the chromatography was added to 0.9 ml of phosphoric acid buffer solution (20 mM, pH7.0) containing methyl 4-bromo-3-oxobutanoate (1.56 mg/ml) and NADPH (0.226 mg/ml) to make a total

volume of 1 ml. After it was maintained at 37 °C for 20 seconds, the absorbance of 340 nm was measured. The NADPH consumption was calculated from the absorbance of 340 nm, thereby recovering the reducing enzyme activity of the fraction.

[0155] (2) The active fraction (A) obtained in the aforementioned step was subjected to SDS polyacryl amide gel electrophoresis according to the method described in "Laemmli, U.K., Nature, (1970) 227, 680". The gel after the electrophoresis was stained with a stain solution, Coomassie brilliant blue G250 (manufactured by Bio-Rad Inc.), and the gel on the stained portion was cut off. This gel was subjected to reductive alkylation by dithiothreitol and acetoamide iodide. After trypsin-treatment, a peptide was extracted from the gel. The extracted peptide was fractionated by HPLC (column: TSK gel ODS-80 = Ts, 2.0mm x 250mm (Toso Co., Ltd.), mobile phase: concentration gradient of 0.1% aqueous trifluoroacetate/acetonitrile = 100/0 → 20/80). A protein sequencer (494cLC) was used to determine the amino acid sequences using the five fractions, which were found to be highly pure according to the TOS-MS. The amino acid sequences determined are shown in SEQ ID NOs: 3, 4, 5, 6 and 7.

[0156] (3) The oligonucleotide primers of SEQ ID NOs: 8, 9, 10, 11, 12, 13 and 14 was synthesized based on the amino acid sequence of SEQ ID NO: 3.

[0157] PCR was conducted by using any one of the oligonucleotide primers of SEQ ID NOs. 8, 9, 10, 11, 12, 13 and 14, and a SK oligonucleotide primer (by Stratagene) as a primer, and the aforementioned cDNA (A) as a template in the reaction solution having the following composition under the following reaction conditions. (Expand High Fidelity PCR System manufactured by Rosche Diagnostic was used.)

Composition of reaction solution

[0158]

cDNA library stock solution	1 µl
dNTP (each 2.5mM-mix)	0.4 µl
Primer (20 pmol/µl)	0.75 µl each
10 x buffer (with MgCl)	5 µl
enz. expandHiFi (3.5 x 10 ³ U/ml)	0.375 µl
Deionized water	41.725 µl

Reaction conditions:

[0159] The reaction vessel containing the reaction solution of the aforementioned composition was set to the PERKIN ELMER-GeneAmp PCR System 2400, and was heated at 97 °C (for 2 minutes). Then a cycle of 97 °C (0.25 min.) → 50 °C (0.5 min.) → 72 °C (1.5 min.) was repeated ten times, followed by a cycle of 97 °C. (0.25 min.) → 55 °C (0.5 min.) → 72 °C (2.5 min.) repeated twenty times, and maintained at 72 °C (7 min.).

[0160] Thereafter, a portion of the PCR reaction solution was subjected to agarose gel electrophoresis, and a band of a DNA fragment of about 740 bp was detected for the following cases: the oligonucleotide primer containing the polynucleotide sequence of SEQ ID NO: 10 and the SK oligonucleotide primer were used as a primer; the oligonucleotide primer containing the polynucleotide sequence of SEQ ID NO: 12 and the SK oligonucleotide primer were used as a primer; and the oligonucleotide primer containing the polynucleotide sequence of SEQ ID NO: 14 and the SK oligonucleotide primer were used as a primer.

[0161] Using each of the PCR reaction solutions where the band of a DNA fragment of about 740 bp was detected, each of the aforementioned DNA fragments of about 740 bp was ligated to the existing "PCR product insertion site" of the p CR2.1-TOPO vector (where TOPO™ TA cloning kit manufactured by Invitrogen was used). *E. coli* DH5 α was transformed with the obtained ligation solution. 30 µl of 4% aqueous solution of 5-bromo-4-chloro-3-indolyl-β-D-galactocide (hereinafter referred to as "X-gal") and 30 µl of 0.1M IPTG were applied onto the LB (1% bacto tryptone, 0.5% bacto yeast-extract and 1% sodium chloride) agar medium containing 50 µg/ml of ampicillin. The transformant obtained was inoculated onto it, and was incubated. Of the colonies formed, each of the white ones was picked up and was inoculated into a sterilized LB medium (2 ml) containing 50 µg/ml of ampicillin. It was incubated in a test tube under shaking (at 30 °C for 24 hours). Plasmid was prepared from each cultivated bacterial cells using the QIAprep Spin Miniprep Kit (manufactured by Qiagen).

[0162] Hereinafter, the plasmid derived from the DNA fragment obtained by PCR using the oligonucleotide primer of SEQ ID NO: 10 and SK oligonucleotide primer as a primer, will be described as "p27-1"; the plasmid derived from the DNA fragment obtained by PCR using the oligonucleotide primer of SEQ ID NO: 12 and SK oligonucleotide primer as a primer, will be described as "p27-2"; and the plasmid derived from the DNA fragment obtained by PCR using the oligonucleotide primer of SEQ ID NO: 14 and SK oligonucleotide primer will be described as "p27-3";

[0163] The polynucleotide sequence of the DNA fragment inserted into each of plasmids p27-1, p27-2 and p27-3 was analyzed, and it was found that the polynucleotide sequences of the inserted DNA fragments were identical except for the primer sequence portions.

[0164] The polynucleotide sequence of the DNA fragment inserted into plasmid p27-1 is shown in SEQ ID NO: 15.

[0165] Analysis of the polynucleotide sequence of the DNA fragment inserted into the plasmid was made as follows: Sequence reaction was carried out according to the Dye Terminator Cycle Sequence FS Ready Reaction Kit (by Perkin Elmer) with each plasmid used as a template, and the polynucleotide sequence of the obtained DNA was analyzed by the DNA sequencer 373A (by Perkin Elmer).

[0166] (4) Oligonucleotide primers containing the polynucleotide sequence of SEQ ID NO: 16 or 17 was synthesized based on the polynucleotide sequence of SEQ ID NO: 15.

[0167] PCR was conducted by using the reaction solution of the following composition and reaction conditions, wherein the oligonucleotide primers containing of SEQ ID NO: 16 and SK oligonucleotide primers (by Stratagene), or the oligonucleotide primer of SEQ ID NO: 17 and T7 oligonucleotide primer (by Stratagene) were used as a primer, and the aforementioned cDNA (A) was employed as a template. (Expand High Fidelity PCR System by Rosche Diagnostic was used.)

Composition of reaction solution:

[0168]

cDNA library stock solution	1 μ l
dNTP (each 2.5mM-mix)	0.4 μ l
Primer (20 pmol/ μ l)	0.75 μ l each
10 x buffer (with MgCl)	5 μ l
enz. expandHiFi (3.5 x 10 ³ U/ml)	0.375 μ l
Deionized water	41.725 μ l

Reaction conditions:

[0169] The reaction vessel containing reaction solution of the aforementioned composition was set to the PERKIN ELMER-GeneAmp PCR System 2400, and was heated at 97 °C (for 2 minutes). Then a cycle of 97 °C (0.25 min.) → 55 °C (0.5 min.) → 72 °C (1.5 min.) was repeated ten times, followed by twenty times a cycle of 97 °C (0.25 min.) → 55 °C (0.5 min.) → 72 °C (2.5 min.). Further, it was maintained at 72 °C for 7 minutes.

[0170] Thereafter, a portion of the PCR reaction solution was subjected to agarose gel electrophoresis. A band of DNA fragment of about 350 bp was detected when the oligonucleotide primer containing the polynucleotide sequence of SEQ ID NO: 16 and the SK oligonucleotide primer were used as a primer. A band of a DNA fragment of about 650 bp was detected when the oligonucleotide primer containing the polynucleotide sequence of SEQ ID NO: 17 and T7 oligonucleotide primer were used as a primer.

[0171] Using the PCR reaction solution containing the DNA fragment of about 350 bp obtained in the aforementioned PCR or the PCR reaction solution containing the DNA fragment of about 650 bp, the aforementioned DNA fragment of about 350 bp and the DNA fragment of about 650 bp were ligated respectively to the existing "PCR product insertion site" of the pCR2.1-TOPO vector (where TOPO™ TA cloning kit by Invitrogen was used). *E. coli* DH5 α was transformed with the obtained ligation solution. 30 μ l of 4% aqueous solution of X-gal and 30 μ l of 0.1M IPTG were applied to the LB agar medium containing 50 μ g/ml of ampicillin. The transformant obtained was inoculated thereon, and was incubated. Of the colonies formed, each of the white ones was picked up and was inoculated into a sterilized LB medium (2 ml) containing 50 μ g/ml of ampicillin. It was incubated under shaking in a test tube (at 30 °C for 24 hours). Plasmid was prepared from each cultivated bacterial cells using the QIAprep Spin Miniprep Kit (by Qiagen).

[0172] Hereinafter, the plasmid derived from the DNA fragment obtained by PCR using the oligonucleotide primer of SEQ ID NO: 16 and SK oligonucleotide primer as a primer, will be denoted by plasmid pBR-1; and the plasmid derived from the DNA fragment obtained by PCR using the oligonucleotide primer of SEQ ID NO: 17 and T7 oligonucleotide primer as primers, will be denoted by pBR-2.

[0173] The polynucleotide sequence of the DNA fragment inserted into each of plasmids pBR-1 and pBR-2 was analyzed. The polynucleotide sequence of the DNA fragment inserted into plasmid pBR-1 is shown in SEQ ID NO: 18. The polynucleotide sequence of the DNA fragment inserted into plasmid pBR-2 is shown in SEQ ID NO: 19.

[0174] Analysis of the polynucleotide sequence of the DNA fragment inserted into the plasmid was made as follows: Sequence reaction was carried out according to the Dye Terminator Cycle Sequence FS Ready Reaction Kit (by Perkin Elmer) with each plasmid used as a template, and the polynucleotide sequence of the obtained DNA was analyzed by

the DNA sequencer 373A (by Perkin Elmer).

[0175] (5) An oligonucleotide primer of SEQ ID NO: 20 was synthesized based on the polynucleotide sequence of SEQ ID NO: 15. An oligonucleotide primer of SEQ ID NO: 21 was synthesized based on the polynucleotide sequence of SEQ ID NO: 19.

[0176] PCR was conducted using the following composition of reaction solution and reaction conditions, wherein the oligonucleotide primer of SEQ ID No: 20 and the oligonucleotide primer of SEQ ID NO: 21 were used as a primer, and the aforementioned cDNA library (A) was employed as a template. (Expand High Fidelity PCR System by Rosche Diagnostic was used.)

Composition of reaction solution:

[0177]

cDNA library stock solution	1 μ l
dNTP (each 2.5mM-mix)	0.4 μ l
Primer (20 pmol/ μ l)	0.75 μ l each
10 x buffer (with MgCl)	5 μ l
enz. expandHiFi (3.5 x 10 ³ U/ml)	0.375 μ l
Deionized water	41.725 μ l

Reaction conditions:

[0178] The reaction vessel containing reaction solution of the aforementioned composition was set to the PERKIN ELMER-GeneAmp PCR System 2400, and was heated at 97 °C (for 2 minutes). Then a cycle of 97 °C (0.25 min.) → 55 °C (0.5 min.) → 72 °C (1.5 min.) was repeated ten times, followed by twenty times a cycle of 97 °C (0.25 min.) → 55 °C (0.5 min.) → 72 °C (2.5 min.). Further, it was maintained at 72 °C for 7 minutes.

[0179] Thereafter, a portion of the PCR reaction solution was subjected to agarose gel electrophoresis. A band of a DNA fragment of about 400 bp was detected.

[0180] Using the PCR reaction solutions containing the DNA fragment of about 400 bp obtained in the aforementioned PCR was ligated to the existing "PCR product insertion site" of the pCR2.1-TOPO vector (TOPO™ TA cloning kit by Invitrogen was used). The obtained ligation solution was utilized to transform *E. coli* DH5 α .

[0181] 30 μ l of 4% aqueous solution of X-gal and 30 μ l of 0.1M IPTG were applied to the LB agar medium containing 50 μ g/ml of ampicillin. The transformant obtained was inoculated thereto, and was incubated. Of the colonies formed, each of the white ones was picked up and was inoculated into a sterilized LB medium (2 ml) containing 50 μ g/ml of ampicillin. It was incubated under shaking in a test tube (at 30 °C for 24 hours). Plasmid was prepared by using the QIAprep Spin Miniprep Kit (by Qiagen) from each cultivated bacterial cells. (Hereinafter, this will be described as plasmid plasmid pBR-3).

[0182] The polynucleotide sequence of the DNA fragment inserted into each of plasmid pBR-3 was analyzed. The polynucleotide sequence of the DNA fragment inserted into plasmid pBR-3 is shown in SEQ ID NO: 22.

[0183] Analysis of the polynucleotide sequence of the DNA fragment inserted into the plasmid was made as follows: Sequence reaction was carried out by using the Dye Terminator Cycle Sequence FS Ready Reaction Kit (by Perkin Elmer) and plasmid pBR-3 as a template, and the polynucleotide sequence of the obtained DNA was analyzed by the DNA sequencer 373A (by Perkin Elmer).

[0184] ORF search was conducted based on the SEQ ID NOs. 18, 19 and 22 to determine the polynucleotide sequence (SEQ ID NO: 28) of the gene coding for the protein capable of preferentially producing methyl (S)-4-bromo-3-hydroxybutanoate by asymmetrically reducing methyl 4-bromo-3-oxobutanoate contained in the *Penicillium citrinum* IFO4631 strain. Further, amino acid sequence of SEQ ID NO: 1 of the protein was determined based on the polynucleotide sequence of SEQ ID NO: 28. Comparison between SEQ ID NO: 1 and SEQ ID NOs. 3, 4, 5, 6, and 7 showed that the amino acid sequence of SEQ ID NOs. 3, 4, 5, 6, and 7 almost coincide with a part of the amino acid sequence of SEQ NO: 1.

Example 2

[0185] (1) The oligonucleotide primer of SEQ ID NO: 23 was synthesized based on the polynucleotide sequence of SEQ ID NO: 18, and that of SEQ ID NO: 24 was synthesized based on the polynucleotide sequence of SEQ ID NO: 19.

[0186] PCR was conducted according to the following composition of reaction solution and reaction conditions, wherein the oligonucleotide primers containing the polynucleotide sequences of SEQ ID NOs. 23 and 24 were used

EP 1 213 354 A2

as primers, and the aforementioned cDNA (A) was employed as a template. (Expand High Fidelity PCR System by Rosche Diagnostic was used.)

Composition of reaction solution:

[0187]

cDNA library stock solution	1 μ l
dNTP (each 2.5mM-mix)	0.4 μ l
Primer (20 pmol/ μ l)	0.75 μ l each
10 x buffer (with MgCl)	5 μ l
enz. expandHiFi (3.5 x 10 ³ U/ml)	0.375 μ l
Deionized water	41.725 μ l

Reaction conditions:

[0188] The vessel containing reaction solution of the aforementioned composition was set to the PERKIN ELMER-GenAmp PCR System 2400, and was heated at 97 °C (for 2 minutes). Then the cycle of 97 °C (0.25 min.) → 55 °C (0.5 min.) → 72 °C (1.5 min.) was repeated ten times, followed by the cycle of 97 °C (0.25 min.) → 55 °C (0.5 min.) → 72 °C (2.5 min.) repeated twenty times. Further, it was maintained at 72 °C for 7 minutes.

[0189] Thereafter, a part of the PCR reaction solution was subjected to agarose gel electrophoresis. A band of a DNA fragment of about 1000 bp was detected.

[0190] Two types of restriction enzymes (NcoI and BamHI) were added to the remaining PCR reaction solution, and the DNA fragment of about 1000 bp was subjected to double digestion, followed by purification of the enzyme digested DNA fragment.

[0191] The plasmid vector pTV118N (Takara Shuzo Co., Ltd.) was digested by two types of restriction enzymes (NcoI and BamHI), and the digested DNA fragments were then purified.

[0192] The DNA fragments subjected to enzyme digestion were mixed and were ligated by T4 DNA ligase. *E. coli* DH5 α was transformed by ligation solution obtained in the above step.

[0193] The obtained transformant was cultured in a LB agar medium containing 50 μ g/ml of ampicillin. Six out of the grown colonies were selected at random. These selected colonies were inoculated into the sterilized LB medium (2 ml) containing 50 μ g/ml of ampicillin. It was incubated under shaking in a test tube (at 30 °C for 24 hours). A plasmid was prepared from each cultured bacterial cell using the QIAprep Spin Miniprep Kit (by Qiagen). A portion of the plasmid obtained in the above step was subjected to double digestion by means of two types of restriction enzymes. Then electrophoresis was carried out and all the plasmids were verified to have about 1000 bp of the aforementioned DNA fragments inserted therein. (This plasmid will be described as plasmid pTRPc hereinafter).

[0194] (2) *E. coli* HB101 was transformed by plasmid pTRPc. The transformant obtained was inoculated in a sterilized LB medium (100 ml) containing 0.1 mM of IPTG and 50 μ g/ml of ampicillin, and cultivated under shaking (at 30 °C for 12 hours). The obtained cultivation solution was subjected to centrifugal separation to obtain 0.4 g of wet bacterial cells. 300 mg of methyl 4-bromo-3-oxobutanoate, 0.4 g of the aforementioned wet bacterial cells, 9 mg of NADP⁺, 750 mg of glucose, 1.2 mg of glucose dehydrogenase (by Amano Pharmaceutical Co., Ltd.), 15 ml of 100 mM phosphoric acid buffer solution (pH 6.5) and 15 ml of butyl acetate were mixed and stirred at 30 °C for 7 hours. During stirring, 2M aqueous solution of sodium carbonate was gradually added so that the pH value of the reaction solution is maintained within a range of 6.5 \pm 0.2. Then the reaction solution was subjected to centrifugal separation to give an organic layer. This organic layer was subjected to content analysis by gas chromatography under the conditions given below. It was found that methyl 4-bromo-3-hydroxybutanoate was obtained in a yield of 98.5% in terms of the methyl 4-bromo-3-oxobutanoate used for reaction. The optical purity of methyl (S)-4-bromo-3-hydroxybutanoate in the organic layer was measured under the conditions given below and found to be 96.1% e. e. This organic layer was concentrated to obtain crude methyl (S)-4-bromo-3-hydroxybutanoate.

Content analysis conditions:

[0195]

Column: HR-20M (0.53 mm x 30 m, 1 μ m) (by Shinwa Kako Co., Ltd.)

Column temperature: 120 °C (5 min) → 3 °C → 150 °C (5 min.) → 10 °C/min → 200 °C (5 min.)

Carrier gas: Helium (flow rate: 20 ml/min.)

Detector: FID

Optical purity measuring conditions:

5 [0196]

Column: G-TA(0.25 cm x 30 m, 0.125 μ m) (by Astech, Ltd.)
 Column Temperature: 110°C(20 min) \rightarrow 5°C/min \rightarrow 180°C (1 min).
 Carrier Gas: Helium (Flow rate: 1 ml/min).
 10 Detector: FID
 Split ratio: 1/50

[0197] The absolute configuration of the product was determined by comparison with an authentic sample of methyl (S)-4-bromo-3-hydroxybutanoate.

15

Example 3

[0198] (1) Plasmid pTRPc was subjected to double digestion by means of two types of restriction enzymes (NcoI and BamHI), and the resulting DNA fragments were purified. Plasmid pTrc99A (by Pharmacia) was subjected to double
 20 digestion by means of two types of restriction enzymes (NcoI and BamHI), and the resulting DNA fragments were purified.

[0199] The digested DNA fragments were mixed and were ligated by T4 DNA ligase. *E. coli* DH5 α was transformed by the ligation solution obtained in the above step.

[0200] The transformant obtained was cultivated on a LB medium containing 50 μ g/ml of ampicillin. Six out of the
 25 grown colonies were selected at random. These selected colonies were inoculated into the sterilized LB medium (2 ml) containing 50 μ g/ml of ampicillin. It was incubated under shaking in a test tube (at 30 °C for 24 hours).

[0201] Plasmids were prepared from each cultivated bacterial cell using the QIAprep Spin Miniprep Kit (by Qiagen). A portion of the plasmid picked up in the above step was subjected to double digestion by means of two types of
 30 restriction enzymes (NcoI and BamHI). Then electrophoresis was carried out and all the plasmids were verified to have the aforementioned DNA fragments of about 1000 bp inserted therein. (This plasmid will be described as plasmid pTrcRPc hereinafter).

[0202] (2) *E. coli* HB101 was transformed by plasmid pTrcRPc.

[0203] The transformant obtained was cultivated in a sterilized LB medium (100 ml) containing 0.1 mM of IPTG and
 35 50 μ g/ml of ampicillin. It was incubated under shaking (at 30 °C for 12 hours). The obtained cultivation solution was subjected to centrifugal separation to give 0.4 g of wet bacterial cells.

[0204] 1500 mg of methyl 4-bromo-3-oxobutanoate, 0.4 g of the aforementioned wet bacterial cells, 18 mg of NADP⁺,
 3000 mg of glucose, 3 mg of glucose dehydrogenase (by Amano Pharmaceutical Co., Ltd.), 15 ml of 100 mM phosphoric
 40 acid buffer solution (pH 6.5) and 15 ml of butyl acetate were mixed and stirred at 30 °C for 7 hours. While stirring, 2M aqueous solution of sodium carbonate was gradually added thereto so that the pH of the reaction solution is maintained with in a range of 6.5 \pm 0.2. Then the reaction solution was subjected to centrifugal separation to obtain an organic
 layer. This organic layer was subjected to content analysis under the conditions given in Example 2. It was found that methyl 4-bromo-3-hydroxybutanoate was obtained in a yield of 99.2 % based on the consumed methyl 4-bromo-3-oxobutanoate. The optical purity of methyl (S)-4-bromo-3-hydroxybutanoate in the organic layer was measured under
 the conditions given in Example 2 and found to be 95.7 % e. e.

45 [0205] This organic layer was further concentrated to give crude methyl (S) 4-bromo-3-hydroxybutanoate.

Example 4

[0206] (1) *Bacillus megaterium* IF012108 strain was cultivated in a sterilized LB medium, thereby obtaining 0.4 g of
 50 bacterial cells. From these bacterial cells, chromosomal DNA (hereinafter referred to as "chromosome DNA (B)") was purified using the Qiagen Genomic Tip (by Qiagen) according to the method described in the Manual attached thereto.

[0207] (2) The oligonucleotide primers of SEQ ID NOs. 25 and 26 were synthesized based on the polynucleotide
 sequence of the glucose dehydrogenase derived from *Bacillus megaterium* IWG3 described in the Journal of Biological
 Chemistry Vol. 264, NO:11, 6381-6385(1989).

55 [0208] PCR was conducted using the primers of SEQ ID NOs. 25 and 26 as primers and the aforementioned DNA
 (B) as a template in the reaction solution of the following composition under the following reaction conditions. (Expand
 High Fidelity PCR System by Rosche Diagnostic was used.)

EP 1 213 354 A2

Composition of reaction solution:

[0209]

Chromosome DNA stock solution	1 μ l
dNTP (each 2.5mM-mix)	0.4 μ l
Primer (20 pmol/ μ l)	0.75 μ l each
10 x buffer (with MgCl)	5 μ l
enz. expandHiFi (3.5 x 10 ³ U/ml)	0.375 μ l
Deionized water	41.725 μ l

Reaction conditions:

[0210] The reaction vessel containing reaction solution of the aforementioned composition was set to the PERKIN ELMER-GeneAmp PCR System 2400, and was heated at 97 °C (for 2 minutes). Then the cycle of 97 °C (0.25 min.) → 55 °C (0.5 min.) → 72 °C (1.5 min.) was repeated ten times, followed by twenty times of a cycle of 97 °C (0.25 min.) → 55 °C (0.5 min.) → 72 °C (2.5 min.). Further, it was maintained at 72 °C for 7 minutes.

[0211] Thereafter, a portion of the PCR reaction solution was subjected to agarose gel electrophoresis. A band of a DNA fragment of about 950 bp was detected.

[0212] Using the PCR reaction solutions obtained in the above step and TOPO™ TA cloning kit by Invitrogen, the DNA fragment of about 950 bp obtained in the aforementioned PCR were ligated to the existing "PCR product insertion site" of the pCR2.1-TOPO vector. The obtained ligation solution was utilized to transform *E. coli* DH5 α .

[0213] 30 μ l of 4% aqueous solution of X-gal and 30 μ l of 0.1M IPTG were applied onto the LB agar medium containing 50 μ g/ml of ampicillin. The transformant obtained was inoculated thereto, and was incubated. Of the colonies formed, one white colony was picked up and was inoculated into a sterilized LB medium (2 ml) containing 50 μ g/ml of ampicillin. It was incubated under shaking in a test tube (at 30 °C for 24 hours). A plasmid was prepared by using the QIAprep Spin Miniprep Kit (by Qiagen) from the cultivated bacterial cells. A portion of the plasmid prepared in the above step was digested with a restriction enzyme (EcoRI). Then electrophoresis was carried out and it was shown that the plasmid has the aforementioned DNA fragment of about 950 bp inserted therein. (This plasmid will be described as plasmid pSDGDH12 hereinafter).

[0214] The polynucleotide sequence of the DNA fragment inserted into plasmid pSDGDH12 was analyzed. The result is shown in SEQ ID NO: 27.

[0215] Analysis of the polynucleotide sequence of the DNA fragment inserted into the plasmid was made as follows: Sequence reaction was carried out according to the Dye Terminator Cycle Sequence FS Ready Reaction Kit (by Perkin Elmer) with plasmid pSDGDH12 as a template, and the polynucleotide sequence of the obtained DNA was analyzed by the DNA sequencer 373A (by Perkin Elmer).

[0216] (3) Plasmid pSDGDH12 was subjected to double digestion by means of two types of restriction enzymes (BamHI and XbaI), and the resulting DNA fragments were purified.

[0217] Plasmid pTrcRbC was subjected to double digestion by means of two types of restriction enzymes (BamHI and XbaI), and the resulting DNA fragments were purified.

[0218] Each of the digested DNA fragments was ligated by T4 DNA ligase. *E. coli* DH5 α was transformed by ligation solution obtained in the above step. The transformant obtained was cultivated on a LB medium containing 50 μ g/ml of ampicillin. Six out of the grown colonies were selected at random. These selected colonies were inoculated into the sterilized LB medium (2 ml) containing 50 μ g/ml of ampicillin. It was incubated under shaking in a test tube (at 30 °C for 24 hours). Plasmid was prepared from each cultivated bacterial cells using the QIAprep Spin Miniprep Kit (by Qiagen). A portion of the plasmid prepared in the above step was subjected to double digestion by means of two types of restriction enzymes (BamHI and XbaI). Then electrophoresis was carried out and all the plasmids were shown to have the inserted DNA fragments of about 1000 bp. (This plasmid will be described as plasmid pTrcRSbG12 hereinafter).

[0219] (4) Plasmid pTrcRSbG12 was used to transform *E. coli* HB101. The transformant obtained was inoculated into a sterilized LB medium (100 ml) containing 0.1 mM IPTG and 50 μ g/ml of ampicillin. It was incubated under shaking (at 30 °C for 12 hours). The incubated solution was subjected to centrifugal separation, thereby obtaining 0.3 g of wet bacterial cells.

[0220] 0.3 g of methyl 4-bromo-3-oxobutanoate, 0.3 g of the aforementioned wet bacterial cells, 9 mg of NADP⁺, 750 mg of glucose, 15 ml of 100 mM phosphate buffer solution (pH 6.5) and 15 ml of butyl acetate were mixed and stirred at 30 °C for 7 hours. While stirring, 2M aqueous solution of sodium carbonate was gradually added so that the pH of the reaction solution is maintained within a range of 6.5 \pm 0.2. Then the reaction solution was subjected to

centrifugal separation to give an organic layer. This organic layer was subjected to content analysis under the conditions given in the Example 2. Methyl 4-bromo-3-hydroxybutanoate was found to have been produced in a yield of 99 % in terms of methyl 4-bromo-3-oxobutanoate used. The optical purity of methyl (S)-4-bromo-3-hydroxybutanoate in the organic layer was measured under the conditions given in Example 2 and was found to be 96% e. e.

[0221] This organic layer was further concentrated to give crude methyl (S)-4-bromo-3-hydroxybutanoate.

Example 5

[0222] (1) The oligonucleotide primer of SEQ ID NO: 29 was synthesized based on the polynucleotide sequence of SEQ ID NO: 19.

[0223] PCR was conducted by using the oligonucleotide primers containing the polynucleotide sequences of SEQ ID NOs. 23 and 29 as primers, and the aforementioned plasmid pTRPc as a template in the reaction solution of the following composition under the following reaction conditions (Expand High Fidelity PCR System by Rosche Diagnostic was used.).

Composition of reaction solution:

[0224]

Plasmid pTRPc solution	1 μ l
dNTP (each 2.5mM-mix)	0.4 μ l
Primer (20 pmol/ μ l)	0.75 μ l each
10 x buffer (with MgCl)	5 μ l
enz. expandHiFi (3.5 x 10 ³ U/ml)	0.375 μ l
Deionized water	41.725 μ l

Reaction conditions:

[0225] The reaction vessel containing reaction solution of the aforementioned composition was set to the PERKIN ELMER-GeneAmp PCR System 2400, and was heated at 97 °C (for 2 minutes). Then a cycle of 97 °C (0.25min.) → 55 °C (0.5 min.) → 72 °C (1.5 min.) was repeated ten times, followed by twenty cycles of 97 °C (0.25 min.) → 55 °C (0.5 min.) → 72 °C (2.5 min.). Further, it was maintained at 72 °C for 7 minutes.

[0226] Thereafter, a part of the PCR reaction solution was subjected to agarose gel electrophoresis. A band of a DNA fragment of about 1000 bp was detected. The remaining PCR reaction solution was purified and digested with two types of restriction enzymes (NcoI and BamHI), and the DNA fragment of about 1000 bp was subjected to double digestion, followed by purification of the enzyme digested DNA fragments.

[0227] The plasmid vector pTV118N (Takara Shuzo Co., Ltd.) was digested by two types of restriction enzymes (NcoI and BamHI), and the digested DNA fragments were then purified.

[0228] The DNA fragments digestion above were mixed and ligated by T4 DNA ligase. *E. coli* DH5 α was transformed by ligation solution obtained in the above step.

[0229] The obtained transformant was cultivated on a LB agar medium containing 50 μ g/ml of ampicillin. Six out of the grown colonies were selected at random. These selected colonies were inoculated into the sterilized LB medium (2 ml) containing 50 μ g/ml of ampicillin. It was incubated under shaking in a test tube (at 30 °C for 24 hours). A plasmid was prepared from each cultured bacterial cell using the QIAprep Spin Miniprep Kit (by Qiagen). A portion of the plasmid prepared in the above step was subjected to double digestion by means of two types of restriction enzymes (NcoI and BamHI). Then electrophoresis was carried out and all the plasmids were shown to have the aforementioned inserted DNA fragments of about 1000 bp. (This plasmid will be described as plasmid pTRPcS hereinafter). The sequence of the DNA fragment inserted into the plasmid pTRPcS was analyzed, and is shown in SEQ ID NO: 30. Sequence analysis of the DNA fragment was conducted by a sequence reaction using Dye Terminator Cycle sequencing FS ready Reaction Kit (Perkin Elmer) and plasmid pTRPcS as a template, and obtained polynucleotide sequence of the DNA was analyzed by DNA Sequencer 373A (Perkin Elmer).

[0230] (2) *E. coli* HB101 was transformed by plasmid pTRPcS. The transformant obtained was inoculated in a sterilized LB medium (100 ml) containing 0.1 mM of IPTG and 50 μ g/ml of ampicillin, and cultivated under shaking (at 30 °C for 12 hours). The obtained cultivation solution was subjected to centrifugal separation to obtain 0.4 g of wet bacterial cells. 300 mg of methyl 4-bromo-3-oxobutanoate, 0.4 g of the aforementioned wet bacterial cells, 9 mg of NADP⁺, 750 mg of glucose, 1.2 mg of glucose dehydrogenase (by Amano Pharmaceutical Co., Ltd.), 15 ml of 100 mM phosphoric acid buffer solution (pH 6.5) and 15 ml of butyl acetate were mixed and stirred at 30 °C for 19 hours. While stirring,

2M aqueous solution of sodium carbonate was gradually added thereto so that the pH value of the reaction solution is maintained within a range of 6.5 ± 0.2 . Then the reaction solution was subjected to centrifugal separation to give an organic layer. This organic layer was subjected to content analysis by gas chromatography as in Example 2. It was found that methyl 4-bromo-3-hydroxybutanoate was obtained in a yield of 97.3 % in terms of the methyl 4-bromo-3-oxobutanoate used for reaction. The optical purity of methyl (S)-4-bromo-3-hydroxybutanoate in the organic layer was measured under the conditions given below and found to be 96.5 % e. e. This organic layer is concentrated to give crude methyl (S)-4-bromo-3-hydroxybutanoate.

Example 6

[0231] (1) The oligonucleotide primers of SEQ ID NOs: 31 and 32 were synthesized based on the polynucleotide sequence of SEQ ID NO: 27.

[0232] PCR was conducted by using the oligonucleotide primers of SEQ ID NOs. 31 and 32 as primers, and the aforementioned DNA (B) as a template in the reaction solution of the following composition under the following reaction conditions (Expand High Fidelity PCR System by Rosche Diagnostic was used.).

Composition of reaction solution:

[0233]

Chromosome DNA stock solution	1 μ l
dNTP (each 2.5mM-mix)	0.4 μ l
Primer (20 pmol/ μ l)	0.75 μ l each
10 x buffer (with MgCl)	5 μ l
enz. expandHiFi (3.5 x 10 ³ U/ml)	0.375 μ l
Deionized water	41.725 μ l

Reaction conditions:

[0234] The reaction vessel containing reaction solution of the aforementioned composition was set to the PERKIN ELMER-GeneAmp PCR System 2400, and was heated at 97 °C (for 2 minutes). Then a cycle of 97 °C (0.25 min.) → 55 °C (0.5 min.) → 72 °C (1.5 min.) was repeated ten times, followed by twenty cycles of 97 °C (0.25 min.) → 55 °C (0.5 min.) → 72 °C (2.5 min.). Further, it was maintained at 72 °C for 7 minutes.

[0235] Thereafter, a part of the PCR reaction solution was subjected to agarose gel electrophoresis. A band of a DNA fragment of about 850 bp was detected. The remaining PCR reaction solution was purified and digested with two types of restriction enzymes (NcoI and BamHI), and the DNA fragment of about 850 bp was subjected to double digestion, followed by purification.

[0236] The plasmid vector pTV118N (Takara Shuzo Co., Ltd.) was digested by two types of restriction enzymes (NcoI and BamHI), and the digested DNA fragments were then purified.

[0237] The digested DNA fragments above were mixed and ligated by T4 DNA ligase. *E. coli* DH5 α was transformed by ligation solution obtained in the above step.

[0238] The obtained transformant was cultivated in a LB agar medium containing 50 μ g/ml of ampicillin. Six out of the grown colonies were selected at random. These selected colonies were inoculated into the sterilized LB medium (2 ml) containing 50 μ g/ml of ampicillin. It was incubated under shaking in a test tube (at 30 °C for 24 hours). A plasmid was obtained from each cultured bacterial cell using the QIAprep Spin Miniprep Kit (by Qiagen). A portion of the plasmid obtained in the above step was subjected to double digestion by means of two types of restriction enzymes (NcoI and BamHI). Then electrophoresis was carried out and all the plasmids were shown to have the aforementioned inserted DNA fragments of about 850 bp. (This plasmid will be described as plasmid pTGDH 12 hereinafter).

[0239] (2) The oligonucleotide primers of SEQ ID NO: 33 was synthesized based on the polynucleotide sequence of plasmid vector pTV118N (by Takara Shuzo, Co., Ltd). PCR reaction was conducted by using the oligonucleotide primers of SEQ ID NO: 32 and NO: 33 as primers and plasmid pTGH12 as a template in the reaction solution of the following composition under the following reaction conditions.

Composition of reaction solution:

[0240]

Plasmid pTGDH12 solution	1 μ l
dNTP (each 2.5mM-mix)	0.4 μ l
Primer (20 pmol/ μ l)	0.75 μ l each
10 x buffer (with MgCl)	5 μ l
enz. expandHiFi (3.5 x 10 ³ U/ml)	0.375 μ l
Deionized water	41.725 μ l

Reaction conditions:

[0241] The reaction vessel containing reaction solution of the aforementioned composition was set to the PERKIN ELMER-GeneAmp PCR System 2400, and was heated at 97 °C (for 2 minutes). Then a cycle of 97 °C (0.25 min.) → 55 °C (0.5 min.) → 72 °C (1.5 min.) was repeated ten times, followed by twenty cycles of 97 °C (0.25 min.) → 55 °C (0.5 min.) → 72 °C (2.5 min.). Further, it was maintained at 72 °C for 7 minutes.

[0242] Thereafter, a portion of the PCR reaction solution was subjected to agarose gel electrophoresis. A band of a DNA fragment of about 1000 bp was detected. The obtained DNA fragment of about 1000 bp was ligated to an existing PCR Product insertion site of pCR2.1-TOPO vector using the PCR solution obtained above and TOPO™ TA cloning kit VER. Eby Invitrogen Co., Ltd, and *E.coli* DH5 α was transformed with the ligation solution.

[0243] 30 μ l of 4% aqueous solution of X-gal and 30 μ l of 0.1M IPTG were applied onto the LB agar medium containing 50 μ g/ml of ampicillin. The transformant obtained was inoculated thereto, and was incubated. Of the colonies formed, one white colony was picked up and was inoculated into a sterilized LB medium (2 ml) containing 50 μ g/ml of ampicillin. It was incubated under shaking in a test tube (at 30 °C for 24 hours). A plasmid was prepared from using the QIAprep Spin Miniprep Kit (by Qiagen) from the cultivated bacterial cells. A portion of the plasmid prepared in the above step was digested with a restriction enzyme (BamHI). Then electrophoresis was carried out and it was shown that the plasmid has the aforementioned inserted DNA fragment of about 1000 bp. (This plasmid will be described as plasmid pCGDH12 hereinafter).

[0244] (3) Plasmid pCGDH12 was subjected to digestion by means of restriction enzymes (BamHI), and the resulting DNA fragment of about 1000bp was purified.

[0245] Plasmid vector pACYC184 (Nippon Gene Co., Ltd.) was subjected to digestion of a restriction enzyme (BamHI), and the resulting DNA fragments were purified, and further dephosphorylated with Alkaline Phosphatase (Takara Shuzo Co., Ltd) to prevent self-ligation.

[0246] The digested DNA fragments above were mixed and ligated by T4 DNA ligase. *E. coli* DH5 α was transformed by ligation solution obtained in the above step. The transformant obtained was cultivated on LB agar medium containing 20 μ g/ml of chloramphenicol. Four out of the grown colonies were selected at random. These selected colonies were inoculated into the sterilized LB medium (2 ml) containing 20 μ g/ml of chloramphenicol. It was incubated under shaking in a test tube (at 30 °C for 24 hours). Plasmid was prepared from each cultivated bacterial cells using the QIAprep Spin Miniprep Kit (by Qiagen). A portion of the plasmid prepared in the above step was subjected to digestion by a restriction enzyme (BamHI). Then electrophoresis was carried out and all the plasmids were shown to have the inserted DNA fragments of about 1000 bp. (This plasmid will be described as plasmid pAGDH12 hereinafter).

[0247] (4) Plasmids pTRPc and pAGDH12 were used to transform *E. coli* HB101. The transformant obtained was inoculated into a sterilized LB medium (100 ml) containing 0.1 mM IPTG and 50 μ g/ml of ampicillin and 20 μ g/ml of chloramphenicol. It was incubated under shaking (at 30 °C for 18 hours). The incubated solution was subjected to centrifugal separation, thereby obtaining 0.4 g of wet bacterial cells.

[0248] 0.3 g of methyl 4-bromo-3-oxobutanoate, 0.4 g of the aforementioned wet bacterial cells, 9 mg of NADP⁺, 750 mg of glucose, 15 ml of 100 mM phosphate buffer solution (pH 6.5) and 15 ml of butyl acetate were mixed and stirred at 30 °C for 19 hours. While stirring, 2M aqueous solution of sodium carbonate was gradually added so that the pH of the reaction solution is maintained within a range of 6.5 \pm 0.2. Then the reaction solution was subjected to centrifugal separation to give an organic layer. This organic layer was subjected to content analysis under the conditions given in the Example 2. Methyl 4-bromo-3-hydroxybutanoate was found to have been produced in a yield of 98.6 % in terms of methyl 4-bromo-3-oxobutanoate used. The optical purity of methyl (S)-4-bromo-3-hydroxybutanoate in the organic layer was measured under the conditions given in Example 2 and was found to be 96.2 % e. e.

[0249] This organic layer is further concentrated to give crude methyl (S)-4-bromo-3-hydroxybutanoate.

Example 7

[0250] Into a 500 ml Sakaguchi flask was placed 100 ml of a sterilized medium (prepared by dissolving 200 g of potato extract and 20 g of dextrose in 1 L of water) and inoculated with cells of *Penicillium citrinum* IFO 4631. Culturing was carried out under aerobic conditions at 30 °C with shaking. The culture was then centrifuged to harvest cells, and the harvested cells were suspended in 5 ml of saline. To this cell suspension was added 300 ml of cold acetone, and the cells were filtered. The resulting cells were dried under vacuum to obtain acetone-dried cells of *Penicillium citrinum* IFO 4631.

[0251] To 15 mg of the acetone-dried cells of *Penicillium citrinum* IFO 4631 described above were added 1.5 ml of butyl acetate in which 15 mg of methyl 4-bromo-3-oxobutanoate was dissolved and 1.5 ml of 100 mM phosphate buffer, pH 6.5, in which 75 mg of glucose, 9 mg of NADP⁺, and 15 U of glucose dehydrogenase were dissolved, and the mixture was shaken at 30 °C for 18 hours. The reaction mixture was then centrifuged to separate the organic layer. The organic layer was analyzed for the content with gas chromatography, and it was confirmed that 5.5 mg of methyl 4-bromo-3-hydroxybutanoate was formed.

Analysis Conditions for Content:

[0252]

Column: HR-20M (0.53 mm x 30 m, 1 μm) (manufactured by Shinwa Kako, Ltd.).
 Column Temperature: 120 °C (5 min.) → 3 °C/min. → 150 °C (5 min.) → 10 °C/min. → 200 °C (5 min.).
 Carrier Gas: Helium (flow rate: 20 ml/min.).
 Detector: FID

[0253] Then, the methyl 4-bromo-3-hydroxybutanoate isolated by concentrating the organic layer under vacuum was dissolved in dichloromethane, and trifluoroacetic anhydride was added. The mixture was allowed to stand at room temperature for one hour. This solution was analyzed under the optical isomer analysis conditions described below, and it was determined that the resulting methyl (S)-4-bromo-3-hydroxybutanoate was 98 %e.e.

Analysis Conditions for Optical Isomers:

[0254]

Column: Chirasil-DEX CB (0.32 mm x 25 m, 0.25 μm) (manufactured by CHROMPACK, Ltd.).
 Column Temperature: 80 °C (20 min.) → 7 °C/min. → 150 °C (5 min.).
 Carrier Gas: Helium (flow rate: 1.25 ml/min.).
 Detector: FID.

[0255] In this case, the absolute configuration of the product was determined by comparison with the authentic sample of methyl (S)-4-bromo-3-hydroxybutanoate.

Example 8

[0256] Into a 500 ml Sakaguchi flask was placed 100 ml of a sterilized medium (prepared by dissolving 200 g of potato extract and 20 g of dextrose in 1 L of water) and inoculated with cells of *Cryptococcus humicola* IFO 1527. Cultivation was carried out under aerobic conditions at 30 °C under shaking. The culture was then centrifuged to harvest cells, and the harvested cells were suspended in 5 ml of saline. To this cell suspension was added 300 ml of cold acetone, and the cells were filtered. The resulting cells were dried under vacuum to obtain acetone-dried cells of *Cryptococcus humicola* IFO 1527.

[0257] To 15 mg of the acetone-dried cells of *Cryptococcus humicola* IFO 1527 were added 1.5 ml of butyl acetate in which 15 mg of methyl 4-bromo-3-oxobutanoate was dissolved and 1.5 ml of 100 mM phosphate buffer, pH 6.5, in which 75 mg of glucose, 9 mg of NADP⁺, and 15 U of glucose dehydrogenase were dissolved, and the mixture was shaken at 30 °C for 18 hours. The reaction mixture was then centrifuged to separate the organic layer. The organic layer was analyzed for the content with gas chromatography, and it was confirmed that 2.0 mg of methyl 4-bromo-3-hydroxybutanoate was formed.

[0258] Next, the methyl 4-bromo-3-hydroxybutanoate isolated by concentrating the organic layer under vacuum was dissolved in dichloromethane, and trifluoroacetic anhydride was added. The mixture was allowed to stand at room temperature for one hour. This solution was analyzed under the same optical isomer analysis conditions as in Example

7, and it was determined that the resulting methyl (S)-4-bromo-3-hydroxybutanoate was 88 % e.e.

Example 9

[0259] Into a 500 ml Sakaguchi flask was placed 100 ml of a sterilized medium (prepared by dissolving 20 g of glucose, 5 g of polypeptone, 3 g of yeast extract, 3 g of meat extract, 1 g of potassium dihydrogen phosphate, 0.5 g of magnesium sulfate heptahydrate, and 2 g of ammonium sulfate in 1 L of water) and inoculated with 0.3 ml cultivated product of *Bacillus alvei* IFO 3343t which had been pre-cultivated in a medium having the same composition. Cultivation was carried out at 30 °C for two days under shaking. The cultivated product was then centrifuged to harvest cells, and the harvested cells were washed with saline and then suspended in 5 ml of saline. To this cell suspension was added 300 ml of cold acetone, and the cells were filtered. The resulting cells were dried under vacuum to obtain acetone-dried cells of *Bacillus alvei* IFO 3343t.

[0260] To 15 mg of the acetone-dried cells of *Bacillus alvei* IFO 3343t described above were added 1.5 ml of butyl acetate in which 15 mg of methyl 4-bromo-3-oxobutanoate was dissolved and 1.5 ml of 100 mM phosphate buffer, pH 6.5, in which 75 mg of glucose, 9 mg of NAD⁺, and 15 U of glucose dehydrogenase were dissolved, and the mixture was shaken at 30 °C for 18 hours. The reaction mixture was then centrifuged to separate the organic layer.

[0261] The content of the product in the organic layer was analyzed with gas chromatography, and it was found that 4.5 mg of methyl 4-bromo-3-hydroxybutanoate was formed.

[0262] Next, the methyl 4-bromo-3-hydroxybutanoate isolated by concentrating the organic layer under reduced pressure was dissolved in dichloromethane, and trifluoroacetic anhydride was added thereto. The mixture was allowed to stand at room temperature for one hour. This solution was analyzed under the same analysis conditions as in Example 7, and it was determined that the purity of the resulting methyl (R)-4-bromo-3-hydroxybutanoate was 96 % e.e.

Example 10

[0263] Into a 30 l jar fermenter was placed 18 l of a sterilized medium (prepared by dissolving 20 g of glucose, 5 g of polypeptone, 3 g of yeast extract, 3 g of meat extract, 1 g of potassium dihydrogen phosphate, 0.5 g of magnesium sulfate heptahydrate, and 2 g of ammonium sulfate in 1 L of water) and added 180 ml culture of *Bacillus alvei* IFO 3343t which had been pre-cultivated in a medium having the same composition. Cultivation was carried out at 30 °C for two days under shaking. The cultivated product was then centrifuged to harvest cells. The harvested cells were washed with saline and then suspended in saline. To this cell suspension was added cold acetone, and the cells were filtered. The resulting cells were dried under vacuum to obtain acetone-dried cells of *Bacillus alvei* IFO 3343t.

[0264] To 750 mg of the acetone-dried cells of *Bacillus alvei* IFO 3343t described above were added 150 mg of ethyl 4,4,4-trifluoro-3-oxobutanoate and 30 ml of 100 mM phosphate buffer, pH 6.5, in which 750 mg of glucose, 9 mg of NAD⁺, and 2.25mg (70 U/mg) of glucose dehydrogenase were dissolved, and the mixture was shaken at 30 °C for 27 hours. Later, ethyl acetate was added, and then centrifuged.

[0265] The organic layer was analyzed with gas chromatography, and it was found that 114 mg of ethyl 4,4,4-trifluoro-3-hydroxybutanoate was formed.

(Analysis Conditions for Content)

[0266]

Column: DB-1 (0.53 mm x 30 m, 1.5 µm).

Column Temperature: 40 °C (5 min.) → 4 °C/min. → 60 °C (0 min.) → 30 °C/min. → 290 °C (2 min.).

Carrier Gas: Helium (flow rate: 20 ml/min.).

Detector: FID.

[0267] Next, the ethyl 4,4,4-trifluoro-3-hydroxybutanoate isolated by concentrating the organic layer under reduced pressure was dissolved in dichloromethane, and trifluoroacetic anhydride was added thereto. The mixture was allowed to stand at room temperature for one hour. This solution was analyzed under the same analysis conditions described below, and it was determined that the purity of the resulting ethyl (R)-4,4,4-trifluoro-3-hydroxybutanoate was 99 % e.e.

(Analysis Conditions for Optical Isomers)

[0268]

Column: GAMMA-DEX 120 (0.25 mm x 30 m, 0.25 µm) (manufactured by SUPELCO, Ltd.).

EP 1 213 354 A2

Column Temperature: 80 °C (5 min.) → 5 °C/min. → 130 °C → 20 °C/min. → 200 °C (5 min.)
 Carrier Gas: Helium (flow rate: 1.25 ml/min.).
 Detector: FID.

5 Example 11

[0269] Into a flask was placed 900 ml of liquid medium (prepared by dissolving 10 g of tryptone, 5 g of yeast extract, and 5 g of sodium chloride in 1000 ml of water, and adding dropwise 1 N aqueous sodium hydroxide solution to adjust the pH to 7.0) and sterilized, and then ampicillin and isopropyl thio-β-D-galactoside (IPTG) were added to make a concentration of 100 µg/ml and 0.4 mM, respectively. This medium was inoculated with 1 ml of the cultivated liquid resulting from cultivation, in the liquid medium having the above-mentioned composition, a transformed *E. coli* strain JM109/pUAR obtained by transforming an *E. coli* strain JM109 by a conventional method using plasmid pUAR containing the DNA shown in SEQ ID NO: 35 (Deposition No. FERM BP-7752 under the Budapest Treaty), and cultivated at 37 °C for 14 hours with shaking. The culture was centrifuged (15000 xg, 15 minutes, 4 °C) to harvest cells, which were suspended in 30 ml of 50 mM monopotassium phosphate-dipotassium phosphate buffer (pH 7.0), followed by centrifugation (15000 xg, 15 minutes, 4 °C) to obtain washed cells.

[0270] To 50 ml of 50 mM monopotassium phosphate-dipotassium phosphate buffer (pH 7.0) containing 5 % of 2-propanol were added 0.1 mmol of NAD⁺ and 6 g of the washed cells described above. To this mixture was added 50 ml of decane containing 70 mg (0.31 mmol) of isopropyl 4-bromo-3-oxobutanoate, and the mixture was stirred at room temperature for a night and day. Celite was then put into the reaction solution, and stirred for a while and filtered off to separate the resultant solution. The aqueous layer was further extracted three times with ethyl acetate, and combined organic layers were concentrated to give 50 mg of isopropyl 4-bromo-3-hydroxybutanoate.

¹H-NMR (CDCl₃) δ(ppm): 1.26 (6H), 2.60 (2H), 3.21 (1H), 3.50 (2H), 4.19-4.28 (1H), 5.02-5.11 (1H).

25 Chemical purity: 99 % (GC).

Optical purity: 77 %e.e. (HPLC, a Daicel chiralcel OD column).

Mobile phase: hexane/isopropanol = 98/2 plus 0.1 % trifluoroacetic acid, 0.5 ml/min., UV 220 nm).

Retention time: 21 minutes.

30 [0271] The following describes Reference Example for producing the authentic sample for determining the optical purity of isopropyl 4-bromo-3-hydroxybutanoate.

Reference Example 2

35 [0272] Twenty-three grams of isopropyl 3-oxobutanoate was dissolved in methylene chloride, and 26 g of bromine was added dropwise while cooling it on ice. The mixture was heated to room temperature and stirred for 8 hours, after which the reaction solution was concentrated to 35 g of isopropyl 4-bromo-3-hydroxybutanoate.

[0273] Four grams of isopropyl 4-bromo-3-hydroxybutanoate was dissolved in 50 ml of ethanol, and while cooling it on ice, a suspension of 4.04 g of sodium boron hydride in 10 ml of ethanol was slowly added dropwise. After adding was completed, acetic acid was added to the reaction solution, water layer and ethyl acetate layer were separated. The organic layer was dried over anhydrous sodium sulfate, and concentrated. The resulting residue was subjected to a column chromatography on silica gel (developing solvent: hexane/ether) to give 1.3 g of isopropyl 4-bromo-3-hydroxybutanoate.

¹H-NMR (CDCl₃) δ(ppm): 1.26 (6H), 2.60 (2H), 3.21 (1H), 3.50 (2H), 4.19-4.28 (1H), 5.02-5.11 (1H).

45 [0274] The resulting isopropyl 4-bromo-3-hydroxybutanoate was analyzed by HPLC using a Daicel chiralcel OD column (mobile phase: hexane/2-propanol = 98/2 plus 0.1 % trifluoroacetic acid, 0.5 ml/min., a UV detector at 220 nm), giving an almost equal area ratio of two peaks at a retention time of 19 and 21 minutes.

Example 12

50 [0275] 1.6 g of calcium chloride (15 mmol) and 0.6 g (8.1 mmol) of calcium hydroxide were dissolved in 3.7 ml of water and stirred at room temperature for 20 minutes. At the same temperature, 2.00 g (10 mmol) of methyl 4-bromo-3-hydroxybutanoate was added dropwise over 5 minutes. After stirring for further ten minutes, the mixture was cooled by ice, and 0.6 g (12 mmol) of sodium cyanide was added thereto. Then, the mixture was stirred at an internal temperature of 25 to 33 °C for 4.5 hours. Then, concentrated hydrochloric acid was added dropwise in the reaction solution, and ethyl acetate was used for extraction five times. The organic layer was concentrated under reduced pressure to get 1.2 g of 4-cyano-3-hydroxybutanoic acid. The purity of the obtained 4-cyano-3-hydroxybutanoic acid was 91% (high-performance liquid chromatography in area percentage).

Example 13

[0276] 35 g of 4-cyano-3-hydroxybutanoic acid was dissolved in 250 g of ethyl acetate, and 41 g of triethylamine and 54 g of diethyl sulfate were added thereto at room temperature. Then, the mixture was stirred at an internal temperature of 55 to 60 °C for 30 minutes. After the reaction solution was left cooled down to room temperature, it was added to a saturated sodium bicarbonate solution, and ethyl acetate was used for extraction. The organic layer was washed with saturated saline solution, and the residue obtained by concentrating under reduced pressure was subjected to distillation under reduced pressure, whereby 24.5 g of ethyl 4-cyano-3-hydroxybutanoate was obtained. The purity of the obtained ethyl 4-cyano-3-hydroxybutanoate was 99% (gas chromatography in area percentage).

Example 14

[0277] 22.8 g (205 mmol) of calcium chloride and 8.4 g (114 mmol) of calcium hydroxide were dissolved in 50 g of water and stirred for 20 minutes. Then, 28.0 g (142 mmol) of methyl 4-bromo-3-hydroxybutanoate was added dropwise at room temperature over 5 minutes. The mixture was stirred at room temperature for 10 minutes and then cooled by ice, and 8.7 g (178 mmol) of sodium cyanide was added thereto. Then, the mixture was further stirred at an internal temperature of 25 to 33 °C for 4.5 hours. Then, concentrated hydrochloric acid was added dropwise, and ethyl acetate was used for extraction five times. The residue that was obtained by concentrating the organic layer under reduced pressure was dissolved in ethyl acetate and dried over anhydrous magnesium sulfate. The solution from which magnesium sulfate was removed through filtration was cooled with ice, and 20.0 g (198 mmol) of triethylamine and 26.5 g (172 mmol) of diethyl sulfate were added thereto. It was stirred for about 30 minutes while gradually heating to room temperature. It was further stirred at an internal temperature of 55 to 63 °C for 40 minutes. Then, the reaction solution was cooled by ice and 50 ml of saturated sodium hydrogen carbonate was added thereto, which was then stirred. This solution was separated, and solution layer was subjected again to extraction with ethyl acetate. Combined organic layers were washed with saturated saline solution dried over anhydrous magnesium sulfate, and concentrated. The residue was subjected to the distillation under reduced pressure and 15.7 g of ethyl 4-cyano-3-hydroxybutanoate was obtained. The purity of the obtained ethyl 4-cyano-3-hydroxybutanoate was 95% (gas chromatography in area percentage).

Example 15

[0278] Into a 500 ml Sakaguchi flask was placed 100 ml of a sterilized medium (prepared by dissolving 200 g of potato extract and 20 g of dextrose in 1 L of water) and inoculated with 0.3 ml cultivated product of *Penicillium citrinum* IFO 4631. Cultivation was aerobically carried out at 30 °C under shaking. The cultivated product was then centrifuged to harvest cells, and the harvested cells were washed with saline. To this cell suspension was added 300 ml of cold acetone, and the cells were collected by filtration. The collected cells were dried at room temperature under vacuum to obtain acetone-treated cells of *Penicillium citrinum* IFO 4631. The same procedure was repeated 15 times to give 150 g of the acetone-treated cells of *Penicillium citrinum* IFO 4631.

[0279] A solution was prepared by dissolving 75 g of glucose, 0.85 g of oxidized form of nicotine adenine dinucleotide phosphate, 0.11 g of glucose dehydrogenase (glucose amino 2; by Amano enzyme) in 1500 g of a phosphate buffer solution (pH 6.5) and 1300 g of butyl acetate and 37 g of methyl 4-bromo-3-oxobutanoate were added thereto, and then 150 g of the acetone-treated cells of *Penicillium citrinum* IFO 4631 described above were added to thereto under stirring. 15 % aqueous sodium carbonate solution was dropwise added thereto so that the pH of the reaction solution is maintained within a range of from 6.5 ± 0.5. After stirring for 24 hours, 90 g of celite were added thereto and filtered under reduced pressure. The organic layer separated from the filtrate was concentrated under reduced pressure to give 28 g of methyl (S)-4-bromo-3-hydroxybutanoate.

Chemical Purity: 88 %, Optical purity: 97 %e.e.

Analysis of Chemical Purity:

[0280]

Column: HR-20M (0.53 mm x 30 m, 1 μm) (manufactured by Shinwa Kako, Ltd.).
 Column Temperature: 120 °C (5 min.) → 3 °C/min. → 150 °C (5 min.) → 10 °C/min. → 200 °C (5 min.).
 Carrier Gas: helium (flow rate: 20 ml/min.).
 Detector: FID.

Analysis of Optical Purity:

[0281]

High performance Liquid chromatography
 Column: Daicel chiralcel OD column (4.6 mm ϕ x 25 cm, 10 μ m)
 Mobile phase: hexane/2-propanol, 0.5 ml/min.,
 Column Temperature: 40°C,
 Detector: UV (220 nm)

[0282] 23 g of calcium chloride and 8.4 g of calcium hydroxide were dissolved in 50 g of water and stirred for 20 minutes. Then, 28.0 g of methyl (S)-4-bromo-3-hydroxybutanoate obtained above was added dropwise at room temperature over 5 minutes. The mixture was stirred at room temperature for 10 minutes and then cooled by ice, and 8.7 g of sodium cyanide was added thereto. Then, the mixture was further stirred at an internal temperature of 25 to 33 °C for 4.5 hours. Then, concentrated hydrochloric acid was added dropwise to the reaction solution to make the pH thereof less than 1, and extracted with ethyl acetate five times. The combined organic layers were concentrated under reduced pressure to give a residue, which was dissolved in ethyl acetate and dried over anhydrous magnesium sulfate. Magnesium sulfate was removed by filtration to give a solution of (R)-4-cyano-3-hydroxybutanoic acid.

[0283] The solution was cooled with ice, and 20.0 g (198 mmol) of triethylamine and 27 g of diethyl sulfate were added thereto. It was stirred for about 30 minutes while gradually heating to room temperature. It was further stirred at an internal temperature of 55 to 63 °C for 40 minutes. Then, the reaction solution was cooled by ice and 50 ml of saturated sodium hydrogen carbonate was added thereto, and separated. Aqueous layer was again extracted with ethyl acetate. Combined organic layers were washed with saturated saline solution dried over anhydrous magnesium sulfate, and concentrated. The residue was concentrated under reduced pressure to give 14 g of ethyl (R)-4-cyano-3-hydroxybutanoate. (Purity: 95 %, Optical purity: 97 %e.e.)

MS (EI, m/z): 157(M⁺),

¹H-NMR (CDCl₃) δ (ppm): 1.29 (t, 6H), 2.57-2.71(m, 4H), 3.57(d, 1H), 4.20(q, 2H), 4.30-4.39(m, 1H).

$[\alpha]_D^{25}$ -28° (c: 1.02, CHCl₃)

"Free text for sequence table"

SEQ ID NO: 8

Oligonucleotide primer designed for PCR

SEQ ID NO: 9

Oligonucleotide primer designed for PCR

SEQ ID NO: 10

Oligonucleotide primer designed for PCR

SEQ ID NO: 11

Oligonucleotide primer designed for PCR

SEQ ID NO: 12

Oligonucleotide primer designed for PCR

SEQ ID NO: 13

Oligonucleotide primer designed for PCR

SEQ ID NO: 14

Oligonucleotide primer designed for PCR

SEQ ID NO: 16

Oligonucleotide primer designed for PCR

SEQ ID NO: 17

Oligonucleotide primer designed for PCR

SEQ ID NO: 20

Oligonucleotide primer designed for PCR

SEQ ID NO: 21

Oligonucleotide primer designed for PCR

SEQ ID NO: 23

Oligonucleotide primer designed for PCR

SEQ ID NO: 24

Oligonucleotide primer designed for PCR

SEQ ID NO: 25

Oligonucleotide primer designed for PCR

SEQ ID NO: 26

EP 1 213 354 A2

Oligonucleotide primer designed for PCR
SEQ ID NO: 27

Oligonucleotide primer designed for PCR
SEQ ID NO: 28

5 Oligonucleotide primer designed for PCR
SEQ ID NO: 29

Oligonucleotide primer designed for PCR
SEQ ID NO: 30

10 Oligonucleotide primer designed for PCR
SEQ ID NO: 31

Oligonucleotide primer designed for PCR
SEQ ID NO: 32

Oligonucleotide primer designed for PCR
SEQ ID NO: 33

15

20

25

30

35

40

45

50

55

SEQUENCE LISTING

<110> Sumitomo Chemical Co., Ltd

<120> PROCESS FOR PRODUCING OPTICALLY ACTIVE 4-HALO-3-HYDROXYBUTANOATE

<130>

<140>

<141>

<160> 27

<170> PatentIn Ver. 2.1

<210> 1

<211> 325

<212> PRT

<213> Penicillium citrinum

<400> 1

Met	Ser	Asn	Gly	Lys	Thr	Phe	Thr	Leu	Ser	Asn	Gly	Val	Lys	Ile	Pro
1				5				10					15		

Gly	Val	Gly	Phe	Gly	Thr	Phe	Ala	Ser	Glu	Gly	Ser	Lys	Gly	Glu	Thr
			20					25					30		

Tyr	Thr	Ala	Val	Thr	Thr	Ala	Leu	Lys	Thr	Gly	Tyr	Arg	His	Leu	Asp
		35					40					45			

Cys	Ala	Trp	Tyr	Tyr	Leu	Asn	Glu	Gly	Glu	Val	Gly	Glu	Gly	Ile	Arg
	50					55					60				

Asp	Phe	Leu	Lys	Glu	Asn	Pro	Ser	Val	Lys	Arg	Glu	Asp	Ile	Phe	Val
65					70					75					80

Cys	Thr	Lys	Val	Trp	Asn	His	Leu	His	Arg	Tyr	Glu	Asp	Val	Leu	Trp
				85					90					95	

EP 1 213 354 A2

Ser Ile Asp Asp Ser Leu Lys Arg Leu Gly Leu Asp Tyr Val Asp Met
 100 105 110
 5
 Phe Leu Val His Trp Pro Ile Ala Ala Glu Lys Asn Gly Gln Gly Glu
 115 120 125
 10
 Pro Lys Ile Gly Pro Asp Gly Lys Tyr Val Ile Leu Lys Asp Leu Thr
 130 135 140
 15
 Glu Asn Pro Glu Pro Thr Trp Arg Ala Met Glu Lys Ile Tyr Glu Asp
 145 150 155 160
 20
 Arg Lys Ala Arg Ser Ile Gly Val Ser Asn Trp Thr Ile Ala Asp Leu
 165 170 175
 25
 Glu Lys Met Ser Lys Phe Ala Lys Val Met Pro His Ala Asn Gln Ile
 180 185 190
 30
 Glu Ile His Pro Phe Leu Pro Asn Glu Glu Leu Val Gln Tyr Cys Phe
 195 200 205
 35
 Ser Lys Asn Ile Met Pro Val Ala Tyr Ser Pro Leu Gly Ser Gln Asn
 210 215 220
 40
 Gln Val Pro Thr Thr Gly Glu Arg Val Ser Glu Asn Lys Thr Leu Asn
 225 230 235 240
 45
 Glu Ile Ala Glu Lys Gly Gly Asn Thr Leu Ala Gln Val Leu Ile Ala
 245 250 255
 50
 Trp Gly Leu Arg Arg Gly Tyr Val Val Leu Pro Lys Ser Ser Asn Pro
 260 265 270
 55
 Lys Arg Ile Glu Ser Asn Phe Lys Ser Ile Glu Leu Ser Asp Ala Asp
 275 280 285
 60
 Phe Glu Ala Ile Asn Ala Val Ala Lys Gly Arg His Phe Arg Phe Val
 290 295 300
 65
 Asn Met Lys Asp Thr Phe Gly Tyr Asp Val Trp Pro Glu Glu Thr Ala
 305 310 315 320

Lys Asn Leu Ser Ala
325

<210> 2

<211> 978

<212> DNA

<213> *Penicillium citrinum*

<220>

<221> CDS

<222> (1).. (978)

<400> 2

atg tct aac gga aag act ttc aca ttg agc aac ggc gtc aag att cct 48
Met Ser Asn Gly Lys Thr Phe Thr Leu Ser Asn Gly Val Lys Ile Pro
1 5 10 15

ggc gtc ggc ttt ggt acc ttc gct agt gaa ggt tcc aag ggc gag acc 96
Gly Val Gly Phe Gly Thr Phe Ala Ser Glu Gly Ser Lys Gly Glu Thr
20 25 30

tat act gct gtc acc act gcc ctg aag acc ggt tac cgt cac ttg gac 144
Tyr Thr Ala Val Thr Thr Ala Leu Lys Thr Gly Tyr Arg His Leu Asp
35 40 45

tgt gcc tgg tac tac ctg aac gag ggt gag gtt ggt gag ggt atc cgt 192
Cys Ala Trp Tyr Tyr Leu Asn Glu Gly Glu Val Gly Glu Gly Ile Arg
50 55 60

gac ttc ctg aag gag aac ccc tcg gtg aag cgt gag gac atc ttc gtc 240
Asp Phe Leu Lys Glu Asn Pro Ser Val Lys Arg Glu Asp Ile Phe Val
65 70 75 80

tgc acc aag gtg tgg aac cac ctc cac cgt tat gag gac gtc ctc tgg 288
Cys Thr Lys Val Trp Asn His Leu His Arg Tyr Glu Asp Val Leu Trp
85 90 95

tcc att gac gac tcc ctg aag cgt ctt gga ctt gac tac gtt gat atg 336
Ser Ile Asp Asp Ser Leu Lys Arg Leu Gly Leu Asp Tyr Val Asp Met
100 105 110

EP 1 213 354 A2

5	ttc ctc gtt cac tgg ccc att gct gcc gag aag aat ggc cag ggt gag Phe Leu Val His Trp Pro Ile Ala Ala Glu Lys Asn Gly Gln Gly Glu	384
	115 120 125	
10	ccc aag att ggc cct gac ggc aaa tac gtc att ctc aag gac ctg acc Pro Lys Ile Gly Pro Asp Gly Lys Tyr Val Ile Leu Lys Asp Leu Thr	432
	130 135 140	
15	gag aac ccc gag ccc aca tgg cgc gct atg gag aag att tat gag gat Glu Asn Pro Glu Pro Thr Trp Arg Ala Met Glu Lys Ile Tyr Glu Asp	480
	145 150 155 160	
20	cgc aag gcc agg tcc att ggt gtc tcc aac tgg acc att gcc gac ctt Arg Lys Ala Arg Ser Ile Gly Val Ser Asn Trp Thr Ile Ala Asp Leu	528
	165 170 175	
25	gag aag atg tcc aag ttc gcc aag gtc atg cct cac gcc aac cag atc Glu Lys Met Ser Lys Phe Ala Lys Val Met Pro His Ala Asn Gln Ile	576
	180 185 190	
30	gag att cac ccc ttc ctg ccc aac gag gag ctg gtg cag tac tgc ttc Glu Ile His Pro Phe Leu Pro Asn Glu Glu Leu Val Gln Tyr Cys Phe	624
	195 200 205	
35	tcc aag aac att atg ccc gtg gcc tac tct cct ctg ggc tcg cag aac Ser Lys Asn Ile Met Pro Val Ala Tyr Ser Pro Leu Gly Ser Gln Asn	672
	210 215 220	
40	cag gtt ccc acc acc ggt gag cgg gtc agc gag aac aag act ctg aac Gln Val Pro Thr Thr Gly Glu Arg Val Ser Glu Asn Lys Thr Leu Asn	720
	225 230 235 240	
45	gag atc gcc gag aag ggc ggc aac acc ctt gct cag gtt ctt att gcc Glu Ile Ala Glu Lys Gly Gly Asn Thr Leu Ala Gln Val Leu Ile Ala	768
	245 250 255	
50	tgg ggt ctg cgc cgt ggc tac gtc gtt ctc ccc aag agc tcc aac ccc Trp Gly Leu Arg Arg Gly Tyr Val Val Leu Pro Lys Ser Ser Asn Pro	816
	260 265 270	
55	aag cgc att gag tcc aac ttc aag agc att gag ctc tcc gat gcc gac Lys Arg Ile Glu Ser Asn Phe Lys Ser Ile Glu Leu Ser Asp Ala Asp	864

EP 1 213 354 A2

	275	280	285	
5	ttt gaa gcc atc aat gcc gtt gcc aag ggt cgt cac ttc cgt ttc gtc			912
	Phe Glu Ala Ile Asn Ala Val Ala Lys Gly Arg His Phe Arg Phe Val			
	290	295	300	
10	aac atg aag gat act ttc gga tat gat gtc tgg ccc gag gag acc gcc			960
	Asn Met Lys Asp Thr Phe Gly Tyr Asp Val Trp Pro Glu Glu Thr Ala			
	305	310	315	320
15	aag aac ctg tct gcg tga			978
	Lys Asn Leu Ser Ala			
	325			
20				
	<210> 3			
	<211> 17			
	<212> PRT			
25	<213> Penicillium citrinum			
	<400> 3			
30	Asn Ile Met Pro Val Ala Tyr Ser Pro Leu Gly Ser Gln Asn Gln Val			
	1 5 10 15			
	Pro			
35				
	<210> 4			
40	<211> 10			
	<212> PRT			
	<213> Penicillium citrinum			
45	<400> 4			
	Ile Pro Gly Val Phe Gly Thr Phe Ala Ser			
	1 5 10			
50				
	<210> 5			
	<211> 17			
	<212> PRT			
55	<213> Penicillium citrinum			

<400> 5

Ser Ile Glu Leu Ser Asp Ala Asp Phe Glu Ala Ile Asn Ala Val Ala
1 5 10 15

Lys

<210> 6

<211> 14

<212> PRT

<213> *Penicillium citrinum*

<400> 6

Met Ile Gly Val Ala Asn Tyr Thr Ile Ala Asp Leu Glu Lys
1 5 10

<210> 7

<211> 14

<212> PRT

<213> *Penicillium citrinum*

<400> 7

Tyr Glu Asp Val Leu Xaa Xaa Ile Asp Asp Ser Leu Lys Arg
1 5 10

<210> 8

<211> 20

<212> DNA

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence: Designed
oligonucleotide primer for PCR

<400> 8

ggaacytgrt tytggsuacc

20

<210> 9
 <211> 20
 <212> DNA
 <213> Artificial Sequence

<220>
 <223> Description of Artificial Sequence:Designed
 oligonucleotide primer for PCR

<400> 9
 tangcnacng gcataatatt 20

<210> 10
 <211> 20
 <212> DNA
 <213> Artificial Sequence

<220>
 <223> Description of Artificial Sequence:Designed
 oligonucleotide primer for PCR

<400> 10
 tangcnacng gcataatggt 20

<210> 11
 <211> 20
 <212> DNA
 <213> Artificial Sequence

<220>
 <223> Description of Artificial Sequence:Designed
 oligonucleotide primer for PCR

<400> 11
 tangcnacng gcatgatatt 20

<210> 12
 <211> 20
 <212> DNA

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence:Designed
oligonucleotide primer for PCR

<400> 12

tangcnacng gcatgatgtt

20

<210> 13

<211> 20

<212> DNA

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence:Designed
oligonucleotide primer for PCR

<400> 13

tangcnacng gcattatatt

20

<210> 14

<211> 20

<212> DNA

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence:Designed
oligonucleotide primer for PCR

<400> 14

tangcnacng gcattatgtt

20

<210> 15

<211> 697

<212> DNA

<213> Escherichia coli

<400> 15

5 ogctctaaaa ctantggatc ccccgggctg caggaattcg gggcgggcgg atccaacgga 60
 aanactttca cactgagcaa cggcgtcaaa attcctggcg tcggctttgg tacctncgct 120
 agtgaagggtt ccaagggcga aacctatnct gctgtcacca ctgccctgaa aaccggttac 180
 cgtncttgg actgtgcctg gtactacctg aacaagggtg aggttgggtga gggtnccgt 240
 gacttcctga aggaaaaccc ctcggtgaag cgtgaggaca tcttctctg caccaagggtg 300
 10 tggaaccacc tccaccgtta tgaggacgtc ctctgttcca ttgacnactc cctgaagcgt 360
 cttggacttg actacgttga tatgttcctc gttcactggc ccattgctgc cgaaaaaat 420
 ggccagggtg agcccaaat tggccctgac ggcaatacn tcttctcaa ggacctgacc 480
 gaaanccna nccacctgg cgcgctatgg aaaaaattt tngatccc aaggccaggt 540
 15 ccattgggtg ttccaattgg accattgocg accttgagaa gatgtccaag ttngccaagg 600
 tnatgcctca cgccaaccag atcgagattc accccttcct gcccaacgag gagctggtgc 660
 agtactgctt ttccaagaac antatgcccg tagcgta 697

20 <210> 16

<211> 21

<212> DNA

<213> Artificial Sequence

25 <220>

<223> Description of Artificial Sequence: Designed
 oligonucleotide primer for PCR

30 <400> 16

ggagggtggtt ccacaccttg g

21

35 <210> 17

<211> 20

40 <212> DNA

<213> Artificial Sequence

<220>

45 <223> Description of Artificial Sequence: Designed
 oligonucleotide primer for PCR

50 <400> 17

caaccagatc gagattcacc

20

55 <210> 18

<211> 331

<212> DNA

<213> Escherichia coli

<400> 18

cgctctaaaa ctantggatc ccccgggctg caggaattcg gcggccgcgg atccttcac 60
 cccatcatgt ctaacggaaa gactttcaca ttgagcaacg gcgtcaagat tcctggcgtc 120
 ggcttttgta ccttcgctag tgaagggtcc aagggcgaga cctatactgc tgtcaccact 180
 gccctgaaga ccggttacgc tcaattggac tgtgcctggt actacctgaa cgagggtgag 240
 gttgggtgagg gtatccgtga cttcctgaag gagaaccctt cgggtgaagcg tgaggacatc 300
 ttcgtctgca ccaagggtgt gaaccacctc c 331

<210> 19

<211> 743

<212> DNA

<213> Escherichia coli

<400> 19

caaccagatc gagattcacc ccttcctgcc caacgaggag ctgggtgcagt actgcttctc 60
 caagaacatt atgcccgagg cctactctcc tctgggctcg cagaaccagg ttcccaccac 120
 cgggtgagcgg gtcagcgaga acaagactct gaacgagatc gccgagaagg gcggcaacac 180
 ccttgctcag gttcttattg cctgggggtct gcgccgtggc tacgtcgttc tcccgaagag 240
 ctccaacccc aagcgcatcg agtccaactt caagagcatt gagctctccg atgccgactt 300
 tgaagccatc aatgccgttg ccaagggtcg tcaattccgt ttctgtcaaca tgaaggatac 360
 tttcggatat gatgtctggc ccgaggagac cgccaagaac ctgtctgcgt gaatctctac 420
 gaaattataa aatnacaccn acnaaaancc aaagcganag gatgatnccc aaaanttttg 480
 agggtttctt ggttgaaaac gtttantgan cccgaantga angaatagat gancttgatt 540
 tctccaaaaa aaaaaaaaaa aaaaacggtc cgcggccgct ccnngggggg gcccggttcc 600
 caattcnccc cttatnattg aattcttttt taanggggnc aaattcncc nnatttcctt 660
 cnanattggn nggcgcctc caaactttcn tcntnaaagg gncccaattc ccccccatt 720
 aantggantt cctntttacc ttt 743

<210> 20

<211> 21

<212> DNA

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence: Designed
 orionucleotide primer for PCR

<400> 20

ccaaggtgtg gaaccacctc c

21

<210> 21

<211> 21

<212> DNA

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence:Designed
oligonucleotide primer for PCR

<400> 21

ccagaggaga gtaggccacg g

21

<210> 22

<211> 417

<212> DNA

<213> Escherichia coli

<400> 22

```

ccaaggtgtg gaaccacctc caccgttatg aggacgtcct ctggtccatt gacgactccc 60
tgaagcgtct tggacttgac tacgttgata tgttcctcgt tcactggccc attgctgccg 120
agaagaatgg ccaggggtgag cccaagattg gccctgacgg caaatacgtc attctcaagg 180
acc+gaccga gaacccccgag cccacatggc gcgctatgga gaagatttat gaggatcgca 240
aggccaggtc cattgggtgtc tccaactgga ccattgccga ccttgagaag atgtccaagt 300
tcgccaaggt catgcctcac gcccaaccaga tcgagattca ccccttcctg cccaacgagg 360
agctgggtgca gtactgcttc tccaagaaca ttatgcccggt ggctactctt cctctgg 417

```

<210> 23

<211> 27

<212> DNA

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence:Designed
oligonucleotide primer for PCR

<400> 23

gccatggcta tgtctaacgg aaagact

27

5

<210> 24

<211> 29

<212> DNA

10

<213> Artificial Sequence

<220>

15

<223> Description of Artificial Sequence:Designed
oligonucleotide primer for PCR

<400> 24

20

cggatccggtt ataatttcgt agagattca

29

<210> 25

25

<211> 21

<212> DNA

<213> Artificial Sequence

<220>

30

<223> Description of Artificial Sequence:Designed
oligonucleotide primer for PCR

<400> 25

35

gatcatcata gcaggagtca t

21

<210> 26

40

<211> 21

<212> DNA

45

<213> Artificial Sequence

<220>

50

<223> Description of Artificial Sequence:Designed
oligonucleotide primer for PCR

<400> 26

55

gaattcaaca ccagtcagct c

21

<210> 27
 <211> 786
 <212> DNA
 <213> Escherichia coli

<220>
 <221> CDS
 <222> (1).. (786)

<400> 27

atg tat aaa gat tta gaa gga aaa gta gtt gtc ata aca ggt tca tct 48
 Met Tyr Lys Asp Leu Glu Gly Lys Val Val Val Ile Thr Gly Ser Ser
 1 5 10 15

acc ggt tta gga aaa gca atg gcg att cgt ttt gcg aca gaa aaa gct 96
 Thr Gly Leu Gly Lys Ala Met Ala Ile Arg Phe Ala Thr Glu Lys Ala
 20 25 30

aaa gta gtt gtg aac tat cgt tcg aaa gaa gaa gaa gct aac agc gtt 144
 Lys Val Val Val Asn Tyr Arg Ser Lys Glu Glu Glu Ala Asn Ser Val
 35 40 45

tta gaa gaa att aaa aaa gtg ggc gga gag gct att gcc gtc aaa ggt 192
 Leu Glu Glu Ile Lys Lys Val Gly Gly Glu Ala Ile Ala Val Lys Gly
 50 55 60

gat gta aca gtt gag tot gat gtg atc aat tta gtt caa tot gct att 240
 Asp Val Thr Val Glu Ser Asp Val Ile Asn Leu Val Gln Ser Ala Ile
 65 70 75 80

aaa gaa ttt gga aag cta gac gtt atg att aat aac gca gga atg gaa 288
 Lys Glu Phe Gly Lys Leu Asp Val Met Ile Asn Asn Ala Gly Met Glu
 85 90 95

aat ccg gtt tcg tot cat gaa atg tot tta agt gat tgg aat aaa gtc 336
 Asn Pro Val Ser Ser His Glu Met Ser Leu Ser Asp Trp Asn Lys Val
 100 105 110

att gat acg aac tta acg gga gca ttt tta ggc agc cgt gaa gog att 384
 Ile Asp Thr Asn Leu Thr Gly Ala Phe Leu Gly Ser Arg Glu Ala Ile
 115 120 125

EP 1 213 354 A2

5	aaa tat ttt gtg gaa aat gat att aag gga aca gtt att aac atg tog Lys Tyr Phe Val Glu Asn Asp Ile Lys Gly Thr Val Ile Asn Met Ser 130 135 140	432
10	agt gtt cac gag aaa att cct tgg cca tta ttt gtt cat tac gca gca Ser Val His Glu Lys Ile Pro Trp Pro Leu Phe Val His Tyr Ala Ala 145 150 155 160	480
15	agt aaa ggc gga atg aag ctc atg acc gaa aca ctt gca tta gaa tac Ser Lys Gly Gly Met Lys Leu Met Thr Glu Thr Leu Ala Leu Glu Tyr 165 170 175	528
20	gct cca aaa ggt att cgt gta aat aac att gga ccg gga gcg att aat Ala Pro Lys Gly Ile Arg Val Asn Asn Ile Gly Pro Gly Ala Ile Asn 180 185 190	576
25	aca ccg att aac gct gag aaa ttt gct gat cct gag cag cgt gca gat Thr Pro Ile Asn Ala Glu Lys Phe Ala Asp Pro Glu Gln Arg Ala Asp 195 200 205	624
30	gta gaa agc atg att cca atg gga tac att gga gag ccg gaa gaa att Val Glu Ser Met Ile Pro Met Gly Tyr Ile Gly Glu Pro Glu Glu Ile 210 215 220	672
35	gca gcg gtt gct gca tgg cta gct tct tca gag gca agt tat gta aca Ala Ala Val Ala Ala Trp Leu Ala Ser Ser Glu Ala Ser Tyr Val Thr 225 230 235 240	720
40	ggg att aca ctc ttt gct gac ggc ggt atg aca cag tac cca tca ttc Gly Ile Thr Leu Phe Ala Asp Gly Gly Met Thr Gln Tyr Pro Ser Phe 245 250 255	768
45	caa gca gga cgc gga taa Gln Ala Gly Arg Gly 260	786
50	<210> 28 <211> 996 <212> DNA	
55	<213> Penicillium citrinum	

<220>

<221> CDS

<222> (1).. (978)

<400> 28

atg tct aac gga aag act ttc aca ttg agc aac ggc gtc aag att cct 48
 Met Ser Asn Gly Lys Thr Phe Thr Leu Ser Asn Gly Val Lys Ile Pro
 1 5 10 15

ggc gtc ggc ttt ggt acc ttc gct agt gaa ggt tcc aag ggc gag acc 96
 Gly Val Gly Phe Gly Thr Phe Ala Ser Glu Gly Ser Lys Gly Glu Thr
 20 25 30

tat act gct gtc acc act gcc ctg aag acc ggt tac cgt cac ttg gac 144
 Tyr Thr Ala Val Thr Thr Ala Leu Lys Thr Gly Tyr Arg His Leu Asp
 35 40 45

tgt gcc tgg tac tac ctg aac gag ggt gag gtt ggt gag ggt atc cgt 192
 Cys Ala Trp Tyr Tyr Leu Asn Glu Gly Glu Val Gly Glu Gly Ile Arg
 50 55 60

gac ttc ctg aag gag aac ccc tcg gtg aag cgt gag gac atc ttc gtc 240
 Asp Phe Leu Lys Glu Asn Pro Ser Val Lys Arg Glu Asp Ile Phe Val
 65 70 75 80

tgc acc aag gtg tgg aac cac ctc cac cgt tat gag gac gtc ctc tgg 288
 Cys Thr Lys Val Trp Asn His Leu His Arg Tyr Glu Asp Val Leu Trp
 85 90 95

tcc att gac gac tcc ctg aag cgt ctt gga ctt gac tac gtt gat atg 336
 Ser Ile Asp Asp Ser Leu Lys Arg Leu Gly Leu Asp Tyr Val Asp Met
 100 105 110

ttc ctc gtt cac tgg ccc att gct gcc gag aag aat ggc cag ggt gag 384
 Phe Leu Val His Trp Pro Ile Ala Ala Glu Lys Asn Gly Gln Gly Glu
 115 120 125

ccc aag att ggc cct gac ggc aaa tac gtc att ctc aag gac ctg acc 432
 Pro Lys Ile Gly Pro Asp Gly Lys Tyr Val Ile Leu Lys Asp Leu Thr
 130 135 140

gag aac ccc gag ccc aca tgg cgc gct atg gag aag att tat gag gat 480

EP 1 213 354 A2

	Glu Asn Pro Glu Pro Thr Trp Arg Ala Met Glu Lys Ile Tyr Glu Asp	
	145	150 155 160
5		
	cgc aag gcc agg tcc att ggt gtc tcc aac tgg acc att gcc gac ctt	528
	Arg Lys Ala Arg Ser Ile Gly Val Ser Asn Trp Thr Ile Ala Asp Leu	
	165	170 175
10		
	gag aag atg tcc aag ttc gcc aag gtc atg cct cac gcc aac cag atc	576
	Glu Lys Met Ser Lys Phe Ala Lys Val Met Pro His Ala Asn Gln Ile	
	180	185 190
15		
	gag att cac ccc ttc ctg ccc aac gag gag ctg gtg cag tac tgc ttc	624
	Glu Ile His Pro Phe Leu Pro Asn Glu Glu Leu Val Gln Tyr Cys Phe	
	195	200 205
20		
	tcc aag aac att atg ccc gtg gcc tac tct cct ctg ggc tcg cag aac	672
	Ser Lys Asn Ile Met Pro Val Ala Tyr Ser Pro Leu Gly Ser Gln Asn	
	210	215 220
25		
	cag gtt ccc acc acc ggt gag cgg gtc agc gag aac aag act ctg aac	720
	Gln Val Pro Thr Thr Gly Glu Arg Val Ser Glu Asn Lys Thr Leu Asn	
	225	230 235 240
30		
	gag atc gcc gag aag ggc ggc aac acc ctt gct cag gtt ctt att gcc	768
	Glu Ile Ala Glu Lys Gly Gly Asn Thr Leu Ala Gln Val Leu Ile Ala	
	245	250 255
35		
	tgg ggt ctg cgc cgt ggc tac gtc gtt ctc ccc aag agc tcc aac ccc	816
	Trp Gly Leu Arg Arg Gly Tyr Val Val Leu Pro Lys Ser Ser Asn Pro	
	260	265 270
40		
	aag cgc att gag tcc aac ttc aag agc att gag ctc tcc gat gcc gac	864
	Lys Arg Ile Glu Ser Asn Phe Lys Ser Ile Glu Leu Ser Asp Ala Asp	
	275	280 285
45		
	ttt gaa gcc atc aat gcc gtt gcc aag ggt cgt cac ttc cgt ttc gtc	912
	Phe Glu Ala Ile Asn Ala Val Ala Lys Gly Arg His Phe Arg Phe Val	
	290	295 300
50		
	aac atg aag gat act ttc gga tat gat gtc tgg ccc gag gag acc gcc	960
	Asn Met Lys Asp Thr Phe Gly Tyr Asp Val Trp Pro Glu Glu Thr Ala	
	305	310 315 320
55		

aag aac ctg tct gcg tga atctctacga aattataa
Lys Asn Leu Ser Ala

996

325

<210> 29

<211> 29

<212> DNA

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence Designed oligonucleotide primer
for PCR

<400> 29

cggatccggtt cacgcagaca gggtttgtg

29

<210> 30

<211> 978

<212> DNA

<213> Penicillium citrinum

<220>

<221> CDS

<222> (1).. (978)

<400> 30

atg tct aac gga aag act ttc aca ttg agc aac ggc gtc aag att cct 48
Met Ser Asn Gly Lys Thr Phe Thr Leu Ser Asn Gly Val Lys Ile Pro
1 5 10 15

ggc gtc ggc ttt ggt acc ttc gct agt gaa ggt tcc aag ggc gag acc 96
Gly Val Gly Phe Gly Thr Phe Ala Ser Glu Gly Ser Lys Gly Glu Thr
20 25 30

tat act gct gtc acc act gcc ctg aag acc ggt tac cgt cac ttg gac 144
Tyr Thr Ala Val Thr Thr Ala Leu Lys Thr Gly Tyr Arg His Leu Asp
35 40 45

tgt gcc tgg tac tac ctg aac gag ggt gag gtt ggt gag ggt atc cgt 192
Cys Ala Trp Tyr Tyr Leu Asn Glu Gly Glu Val Gly Glu Gly Ile Arg
50 55 60

EP 1 213 354 A2

5	gac ttc ctg aag gag aac ccc tcg gtg aag cgt gag gac atc ttc gtc Asp Phe Leu Lys Glu Asn Pro Ser Val Lys Arg Glu Asp Ile Phe Val 65 70 75 80	240
10	tgc acc aag gtg tgg aac cac ctc cac cgt tat gag gac gtc ctc tgg Cys Thr Lys Val Trp Asn His Leu His Arg Tyr Glu Asp Val Leu Trp 85 90 95	288
15	tcc att gac gac tcc ctg aag cgt ctt gga ctt gac tac gtt gat atg Ser Ile Asp Asp Ser Leu Lys Arg Leu Gly Leu Asp Tyr Val Asp Met 100 105 110	336
20	ttc ctc gtt cac tgg ccc att gct gcc gag aag aat ggc cag ggt gag Phe Leu Val His Trp Pro Ile Ala Ala Glu Lys Asn Gly Gln Gly Glu 115 120 125	384
25	ccc aag att ggc cct gac ggc aaa tac gtc att ctc aag gac ctg acc Pro Lys Ile Gly Pro Asp Gly Lys Tyr Val Ile Leu Lys Asp Leu Thr 130 135 140	432
30	gag aac ccc gag ccc aca tgg cgc gct atg gag aag att tat gag gat Glu Asn Pro Glu Pro Thr Trp Arg Ala Met Glu Lys Ile Tyr Glu Asp 145 150 155 160	480
35	cgc aag gcc agg tcc att ggt gtc tcc aac tgg acc att gcc gac ctt Arg Lys Ala Arg Ser Ile Gly Val Ser Asn Trp Thr Ile Ala Asp Leu 165 170 175	528
40	gag aag atg tcc aag ttc gcc aag gtc atg cct cac gcc aac cag atc Glu Lys Met Ser Lys Phe Ala Lys Val Met Pro His Ala Asn Gln Ile 180 185 190	576
45	gag att cac ccc ttc ctg ccc aac gag gag ctg gtg cag tac tgc ttc Glu Ile His Pro Phe Leu Pro Asn Glu Glu Leu Val Gln Tyr Cys Phe 195 200 205	624
50	tcc aag aac att atg ccc gtg gcc tac tct cct ctg ggc tcg cag aac Ser Lys Asn Ile Met Pro Val Ala Tyr Ser Pro Leu Gly Ser Gln Asn 210 215 220	672
55	cag gtt ccc acc acc ggt gag cgg gtc agc gag aac aag act ctg aac	720

EP 1 213 354 A2

Gln Val Pro Thr Thr Gly Glu Arg Val Ser Glu Asn Lys Thr Leu Asn
225 230 235 240

gag atc gcc gag aag ggc ggc aac acc ctt gct cag gtt ctt att gcc 768
Glu Ile Ala Glu Lys Gly Gly Asn Thr Leu Ala Gln Val Leu Ile Ala
245 250 255

tgg ggt ctg cgc cgt ggc tac gtc gtt ctc ccc aag agc tcc aac ccc 816
Trp Gly Leu Arg Arg Gly Tyr Val Val Leu Pro Lys Ser Ser Asn Pro
260 265 270

aag cgc att gag tcc aac ttc aag agc att gag ctc tcc gat gcc gac 864
Lys Arg Ile Glu Ser Asn Phe Lys Ser Ile Glu Leu Ser Asp Ala Asp
275 280 285

ttt gaa gcc atc aat gcc gtt gcc aag ggt cgt cac ttc cgt ttc gtc 912
Phe Glu Ala Ile Asn Ala Val Ala Lys Gly Arg His Phe Arg Phe Val
290 295 300

aac atg aag gat act ttc gga tat gat gtc tgg ccc gag gag acc gcc 960
Asn Met Lys Asp Thr Phe Gly Tyr Asp Val Trp Pro Glu Glu Thr Ala
305 310 315 320

aag aac ctg tct gcg tga 978
Lys Asn Leu Ser Ala
325

<210> 31
<211> 27
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence Designed oligonucleotide primer
for PCR

<400> 31
gccatggcta tgtataaaga tttagaa 27

<210> 32
<211> 23

<212> DNA

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence Designed oligonucleotide primer for PCR

<400> 32

cggatccggtt atccgcgtcc tgc

23

<210> 33

<211> 28

<212> DNA

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence Designed oligonucleotide primer for PCR

<400> 33

cggatccgag cgcccaatac gcaaaccg

28

<210> 34

<211> 385

<212> PRT.

<213> Corynebacterium sp.

<400> 34

Met Lys Ala Ile Gln Tyr Thr Arg Ile Gly Ala Glu Pro Glu Leu Thr

1

5

10

15

Glu Ile Pro Lys Pro Glu Pro Gly Pro Gly Glu Val Leu Leu Glu Val

20

25

30

Thr Ala Ala Gly Val Cys His Ser Asp Asp Phe Ile Met Ser Leu Pro

35

40

45

Glu Glu Gln Tyr Thr Tyr Gly Leu Pro Leu Thr Leu Gly His Glu Gly

50

55

60

49

5	Ala Gly Lys Val Ala Val Gly Glu Gly Val Glu Gly Leu Asp Ile	65	70	75	80
10	Gly Thr Asn Val Val Val Tyr Gly Pro Trp Gly Cys Gly Asn Cys Trp	85	90	95	
15	His Cys Ser Gln Gly Leu Glu Asn Tyr Cys Ser Arg Ala Gln Glu Leu	100	105	110	
20	Gly Ile Asn Pro Pro Gly Leu Gly Ala Pro Gly Ala Leu Ala Glu Phe	115	120	125	
25	Met Ile Val Asp Ser Pro Arg His Leu Val Pro Ile Gly Asp Leu Asp	130	135	140	
30	Pro Val Lys Thr Val Pro Leu Thr Asp Ala Gly Leu Thr Pro Tyr His	145	150	155	160
35	Ala Ile Lys Arg Ser Leu Pro Lys Leu Arg Gly Gly Ser Tyr Ala Val	165	170		175
40	Val Ile Gly Thr Gly Gly Leu Gly His Val Ala Ile Gln Leu Leu Arg	180	185		190
45	His Leu Ser Ala Ala Thr Val Ile Ala Leu Asp Val Ser Ala Asp Lys	195	200		205
50	Leu Glu Leu Ala Thr Lys Val Gly Ala His Glu Val Val Leu Ser Asp	210	215		220
55	Lys Asp Ala Ala Glu Asn Val Arg Lys Ile Thr Gly Ser Gln Gly Ala	225	230	235	240
60	Ala Leu Val Leu Asp Phe Val Gly Tyr Gln Pro Thr Ile Asp Thr Ala	245	250		255
65	Met Ala Val Ala Gly Val Gly Ser Asp Val Thr Ile Val Gly Ile Gly	260	265		270
70	Asp Gly Gln Ala His Ala Lys Val Gly Phe Phe Gln Ser Pro Tyr Glu	275	280		285

EP 1 213 354 A2

5 Ala Ser Val Thr Val Pro Tyr Trp Gly Ala Arg Asn Glu Leu Ile Glu
290 295 300

10 Leu Ile Asp Leu Ala His Ala Gly Ile Phe Asp Ile Gly Gly Gly Asp
305 310 315 320

15 Leu Gln Ser Arg Gln Arg Cys Arg Ser Val Ser Thr Thr Gly Cys Arg
325 330 335

20 Asn Ala Gln Arg Pro Cys Gly Cys Gly Pro Trp Ser Val Val Pro Thr
340 345 350

25 Ala Val Glu Arg Gln Arg Lys Asn Thr Asp Ala Arg Pro Asn Ser Ile
355 360 365

30 Arg Pro Gly Ile Ser Val Arg Asn Ser Val Cys Ala Ser Cys Thr Pro
370 375 380

35 Arg
385

35 <210> 35
<211> 1158
<212> DNA
<213> Corynebacterium sp.

40 <220>
<221> CDS
<222> (1).. (1158)

45 <400> 35
atg aag gcg atc cag tac acg cga atc ggc gcg gaa ccc gaa ctc acg 48
Met Lys Ala Ile Gln Tyr Thr Arg Ile Gly Ala Glu Pro Glu Leu Thr
1 5 10 15

50 gag att ccc aaa ccc gag ccc ggt cca ggt gaa gtg ctc ctg gaa gtc 96
Glu Ile Pro Lys Pro Glu Pro Gly Pro Gly Glu Val Leu Leu Glu Val
20 25 30

55 acc gct gct ggc gtc tgc cac tcg gac gac ttc atc atg agc ctg ccc 144

EP 1 213 354 A2

Thr Ala Ala Gly Val Cys His Ser Asp Asp Phe Ile Met Ser Leu Pro
 35 40 45

gaa gag cag tac acc tac ggc ctt ccg ctc acg ctc ggc cac gaa ggc 192
 Glu Glu Gln Tyr Thr Tyr Gly Leu Pro Leu Thr Leu Gly His Glu Gly
 50 55 60

gca ggc aag gtc gcc gcc gtc ggc gag ggt gtc gaa ggt ctc gac atc 240
 Ala Gly Lys Val Ala Ala Val Gly Glu Gly Val Glu Gly Leu Asp Ile
 65 70 75 80

gga acc aat gtc gtc gtc tac ggg cct tgg ggt tgc ggc aac tgt tgg 288
 Gly Thr Asn Val Val Val Tyr Gly Pro Trp Gly Cys Gly Asn Cys Trp
 85 90 95

cac tgc tca caa gga ctc gag aac tat tgc tct cgc gcc caa gaa ctc 336
 His Cys Ser Gln Gly Leu Glu Asn Tyr Cys Ser Arg Ala Gln Glu Leu
 100 105 110

gga atc aat cct ccc ggt ctc ggt gca ccc ggc gcg ttg gcc gag ttc 384
 Gly Ile Asn Pro Pro Gly Leu Gly Ala Pro Gly Ala Leu Ala Glu Phe
 115 120 125

atg atc gtc gat tct cct cgc cac ctt gtc ccg atc ggt gac ctc gac 432
 Met Ile Val Asp Ser Pro Arg His Leu Val Pro Ile Gly Asp Leu Asp
 130 135 140

ccg gtc aag acg gtg ccg ctg acc gac gcc ggt ctg acg ccg tat cac 480
 Pro Val Lys Thr Val Pro Leu Thr Asp Ala Gly Leu Thr Pro Tyr His
 145 150 155 160

gcg atc aag cgt tct ctg ccg aaa ctt cgc gga ggc tcg tac gcg gtt 528
 Ala Ile Lys Arg Ser Leu Pro Lys Leu Arg Gly Gly Ser Tyr Ala Val
 165 170 175

gtc att ggt acc ggc ggt ctc ggc cac gtc gct att cag ctc ctc cgc 576
 Val Ile Gly Thr Gly Gly Leu Gly His Val Ala Ile Gln Leu Leu Arg
 180 185 190

cac ctc tcg gcg gca acg gtc atc gct ttg gac gtg agc gcg gac aag 624
 His Leu Ser Ala Ala Thr Val Ile Ala Leu Asp Val Ser Ala Asp Lys
 195 200 205

EP 1 213 354 A2

5	ctc gaa ctg gca acc aag gta ggc gct cac gaa gtg gtt ctg tcc gac Leu Glu Leu Ala Thr Lys Val Gly Ala His Glu Val Val Leu Ser Asp 210 215 220	672
10	aag gac gcg gcc gag aac gtc cgc aag atc act gga agt caa ggc gcc Lys Asp Ala Ala Glu Asn Val Arg Lys Ile Thr Gly Ser Gln Gly Ala 225 230 235 240	720
15	gca ttg gtt ctc gac ttc gtc ggc tac cag ccc acc atc gac acc gcg Ala Leu Val Leu Asp Phe Val Gly Tyr Gln Pro Thr Ile Asp Thr Ala 245 250 255	768
20	atg gct gtc gcc ggc gtc gga tca gac gtc acg atc gtc ggg atc ggg Met Ala Val Ala Gly Val Gly Ser Asp Val Thr Ile Val Gly Ile Gly 260 265 270	816
25	gac ggc cag gcc cac gcc aaa gtc ggg ttc ttc caa agt cct tac gag Asp Gly Gln Ala His Ala Lys Val Gly Phe Phe Gln Ser Pro Tyr Glu 275 280 285	864
30	gct tcg gtg aca gtt ccg tat tgg ggt gcc cgc aac gag ttg atc gaa Ala Ser Val Thr Val Pro Tyr Trp Gly Ala Arg Asn Glu Leu Ile Glu 290 295 300	912
35	ttg atc gac ctc gcc cac gcc ggc atc ttc gac atc ggc ggt gga gac Leu Ile Asp Leu Ala His Ala Gly Ile Phe Asp Ile Gly Gly Gly Asp 305 310 315 320	960
40	ctt cag tct cga caa cgg tgc cga agc gta tcg acg act ggc tgc cgg Leu Gln Ser Arg Gln Arg Cys Arg Ser Val Ser Thr Thr Gly Cys Arg 325 330 335	1008
45	aac gct cag cgg ccg tgc ggt tgt ggt ccc tgg tct gta gta ccg aca Asn Ala Gln Arg Pro Cys Gly Cys Gly Pro Trp Ser Val Val Pro Thr 340 345 350	1056
50	gog gta gaa cga cag cgg aaa aac act gat gcc cgg ccg aat tcg att Ala Val Glu Arg Gln Arg Lys Asn Thr Asp Ala Arg Pro Asn Ser Ile 355 360 365	1104
55	cgg ccg ggc atc agt gtc aga aat tcg gtg tgc gct agc tgc acg cct	1152

Arg Pro Gly Ile Ser Val Arg Asn Ser Val Cys Ala Ser Cys Thr Pro
 370 375 380

cga tga

1158

Arg

385

Claims

1. A polynucleotide comprising:

- a) a polynucleotide sequence coding for an amino acid sequence represented by SEQ ID NO: 1;
- b) a polynucleotide sequence that hybridizes, under stringent conditions, with a polynucleotide sequence coding for an amino acid sequence represented by SEQ ID NO: 1, the amino acid sequence being an amino acid sequence of a protein capable of preferentially producing (S)-4-bromo-3-hydroxybutanoate by asymmetrically reducing 4-bromo-3-oxobutanoate; or
- c) a polynucleotide sequence represented by SEQ ID NO: 2.

2. A DNA construct comprising a promoter in operative linkage with a polynucleotide as defined in claim 1.

3. A recombinant vector comprising a polynucleotide as defined in claim 1 or a DNA construct as defined in claim 2.

4. A transformant comprising a polynucleotide as defined in claim 1, a DNA construct as defined in claim 2, or a vector as defined in claim 3.

5. A transformant according to claim 4, wherein the transformant is a microorganism.

6. A transformant according to claim 5, wherein the microorganism is *E. coli*.

7. A process for producing a transformant, which comprises the step of introducing a recombinant vector as defined in claim 3 into a host cell.

8. A recombinant vector comprising:

- A) a polynucleotide as defined in claim 1 or a DNA construct as defined in claim 2; and
- B) a polynucleotide coding for an enzyme capable of converting oxidized β -nicotinamide-adenine dinucleotide phosphate into a reduced form.

9. A recombinant vector according to claim 8, wherein the enzyme capable of converting oxidized β -nicotinamide-adenine dinucleotide phosphate into a reduced form is glucose dehydrogenase.

10. A transformant comprising a vector according to claim 8 or 9.

11. A transformant according to claim 10, wherein the transformant is a microorganism.

12. A transformant according to claim 11, wherein the microorganism is *E. coli*.

13. A transformant comprising:

- A) a polynucleotide as defined in claim 1 or a DNA construct as defined in claim 2; and
- B) a polynucleotide coding for an enzyme capable of converting oxidized β -nicotinamide-adenine dinucleotide phosphate into a reduced form.

14. A protein comprising:

- i) an amino acid sequence of SEQ ID NO: 1;
- ii) an amino acid sequence encoded by a polynucleotide sequence that hybridizes under stringent conditions with a polynucleotide sequence of SEQ ID NO: 2 coding for an amino acid sequence of a protein capable of preferentially producing (S)-4-bromo-3-hydroxybutanoate by asymmetrically reducing 4-bromo-3-oxobutanoate; or
- iii) an amino acid sequence of SEQ ID NO: 1, wherein one or more amino acids are deleted, replaced or added, said amino acid sequence being an amino acid sequence of a protein capable of preferentially producing (S)-4-bromo-3-hydroxybutanoate by asymmetrically reducing 4-bromo-3-oxobutanoate.

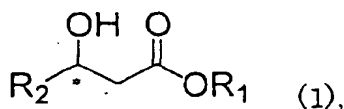
15. A process for producing (S)-4-halo-3-hydroxybutanoate, which comprises reacting a 4-halo-3-oxobutanoic acid ester with a protein as defined in claim 14, a transformant which produces said protein or a treated product thereof.

16. A process according to claim 15, which comprises allowing the coexistence of an enzyme capable of converting oxidized β -nicotinamide-adenine dinucleotide phosphate into a reduced form.

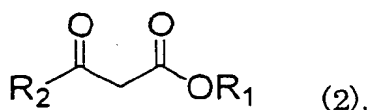
17. A process according to claim 16, wherein the enzyme capable of converting oxidized β -nicotinamide-adenine dinucleotide phosphate into a reduced form is a glucose dehydrogenase.

18. A process according to any one of claims 15 to 17, wherein the 4-halo-3-oxobutanoic acid ester is contacted with a transformant as defined in any one of claims 10 to 13 or a treated product thereof.

19. A process according to any one of claims 15 to 18, wherein the 4-halo-3-oxobutanoic acid ester is represented by formula (1):

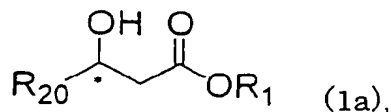


wherein R_1 represents an alkyl group, and R_2 represents a methyl group which is substituted with a halogen atom, which process comprises reacting a 4-halo-3-oxobutanoic acid ester of formula (2):

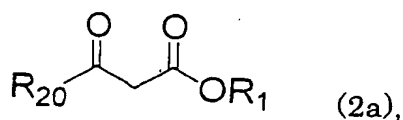


wherein R_1 and R_2 are as defined above.

20. A process for producing an optically active 3-hydroxybutanoic acid ester of formula (1a):

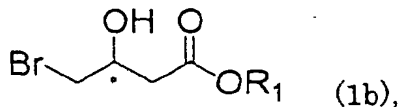


wherein R_1 represents an alkyl group, and R_{20} represents a methyl group which may be substituted with a halogen atom, which process comprises reacting a 3-oxobutanoic acid ester of formula (2a):

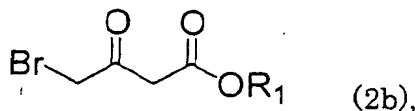


wherein R_1 and R_{20} are as defined above, with whole cells of a microorganism or a treated product thereof, which microorganism belongs to *Penicillium citrinum*, *Cryptococcus humicola*, or *Bacillus alvei* and is capable of asymmetrically reducing the oxo group at the 3-position of the compound of formula (2a) to the corresponding 3-hydroxy group.

21. A process according to claim 20, wherein R_2 represents a halomethyl group.
22. A process according to claim 20 or 21, wherein the microorganism is a strain selected from *Penicillium citrinum* (IFO4631), *Cryptococcus humicola* (IFO1527), and *Bacillus alvei* (IFO33431).
23. A process for producing an optically active 4-bromo-3-hydroxybutanoate of formula (1b):



wherein R_1 represents a (C2-C8)alkyl group, which process comprises reacting a 4-bromo-3-oxobutanoate of formula (2b):

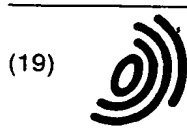


wherein R_1 is as defined above, with an enzyme which has an amino acid sequence comprising:

- iv) an amino acid sequence of SEQ ID NO: 34;
- v) an amino acid sequence encoded by a polynucleotide sequence that hybridizes, under stringent conditions, with a polynucleotide sequence of SEQ ID NO: 34, wherein said amino acid sequence is an amino acid sequence of a protein capable of preferentially producing optically active 4-bromo-3-hydroxybutanoate by asymmetrically reducing 4-bromo-3-oxobutanoate; and
- vi) an amino acid sequence of SEQ ID NO: 3, wherein one or more amino acids are deleted, replaced or added, said amino acid sequence being an amino acid sequence of a protein capable of preferentially producing optically active 4-bromo-3-hydroxybutanoate by asymmetrically reducing 4-bromo-3-oxobutanoate.

24. A process for producing 4-cyano-3-hydroxybutanoic acid, which comprises reacting a 4-bromo-3-hydroxybutanoic acid ester with a metal cyanide in the presence of an alkaline earth metal hydroxide and an alkaline earth metal halogenide.
25. A process according to claim 24, which further comprises the step of reacting the 4-cyano-3-hydroxybutanoic acid with dialkyl sulfate to produce a 4-cyano-3-hydroxybutanoic acid alkyl ester.
26. A process according to claim 24 or 25, wherein the alkaline earth metal hydroxide is calcium hydroxide, and the alkaline earth metal halogenide is calcium chloride.
27. A process according to any one of claims 24 to 26, wherein the 4-bromo-3-hydroxybutanoic acid ester is (C1-C8) alkyl 4-bromo-3-hydroxybutanoate, and the dialkyl sulfate is dimethyl or diethyl sulfate.

28. A process according to any one of claims 24 to 27, wherein the 4-bromo-3-hydroxybutanoic acid ester and 4-cyano-3-hydroxybutanoic acid are optically active compounds.
29. A process according to any one of claims 24 to 28, wherein the 4-bromo-3-hydroxybutanoic acid ester is an (S)-4-bromo-3-hydroxybutanoic acid ester and the 4-cyano-3-hydroxybutanoic acid is (R)-4-cyano-3-hydroxybutanoic acid.
30. A process for producing (R)-4-cyano-3-hydroxybutanoic acid, which comprises producing an (S)-4-bromo-3-hydroxybutanoic acid ester by asymmetrically reducing the 4-bromo-3-oxobutanoic acid ester, and reacting the (S)-4-bromo-3-hydroxybutanoic acid ester with a metal cyanide in the presence of an alkaline earth metal hydroxide and an alkaline earth metal halogenide.
31. A process according to claim 30, wherein the asymmetrical reduction is conducted by a microorganism or treated product thereof capable of asymmetrically reducing the 4-bromo-3-oxobutanoic acid ester to (S)-4-bromo-3-hydroxybutanoic acid ester.
32. A process according to claim 31, wherein the microorganism is a microorganism belonging to *Penicillium citrinum*.
33. A process according to any one of claims 30 to 32, wherein the (S)-4-bromo-3-hydroxybutanoic acid ester and 4-bromo-3-oxobutanoic acid ester are (C1-C8)alkyl esters.
34. A process according to claim 32, wherein the microorganism is a strain *Penicillium citrinum* (IFO4631).
35. A process according to any one of claims 30 to 34, wherein the alkaline earth metal hydroxide is calcium hydroxide and the alkaline earth metal halogenide is calcium chloride.
36. A process according to any one of claims 30 to 35, which further comprises the step of reacting (R)-4-cyano-3-hydroxybutanoic acid with dialkyl sulfate to produce an (R)-4-cyano-3-hydroxybutanoic acid alkyl ester.
37. A process according to claim 36, wherein the alkyl group of the dialkyl sulfate is a methyl or ethyl group.



Europäisches Patentamt
European Patent Office
Office européen des brevets



(11) **EP 1 213 354 A3**

(12) **EUROPEAN PATENT APPLICATION**

(88) Date of publication A3:
05.02.2003 Bulletin 2003/06

(51) Int Cl.7: **C12N 15/53**, C12N 9/04,
C12N 1/21, C12P 7/42,
C12P 7/62

(43) Date of publication A2:
12.06.2002 Bulletin 2002/24

(21) Application number: **01310251.2**

(22) Date of filing: **07.12.2001**

(84) Designated Contracting States:
AT BE CH CY DE DK ES FI FR GB GR IE IT LI LU
MC NL PT SE TR
Designated Extension States:
AL LT LV MK RO SI

(30) Priority: **07.12.2000 JP 2000372704**
15.01.2001 JP 2001006144
02.02.2001 JP 2001026594
11.06.2001 JP 2001175175

(83) Declaration under Rule 28(4) EPC (expert
solution)

(71) Applicant: **Sumitomo Chemical Company,**
Limited
Chuo-ku Osaka 541-8550 (JP)

(72) Inventors:

- **Asako, Hiroyuki**
Minoo-shi, Osaka (JP)
- **Matsumura, Kenji**
Minoo-shi, Osaka (JP)
- **Shimizu, Masatoshi**
Toyonaka-shi, Osaka (JP)
- **Ito, Nobuya**
Tayama-shi, Toyama (JP)
- **Wakita, Ryuhei**
Toyonaka-shi, Osaka (JP)

(74) Representative: **Cresswell, Thomas Anthony**
J.A. KEMP & CO.
14 South Square
Gray's Inn
London WC1R 5JJ (GB)

(54) **Process for producing optically active 4-halo-3-hydroxybutanoate**

(57) There are provided a polynucleotide sequence coding for an amino acid sequence capable of preferentially producing (S)-4-bromo-3-hydroxybutanoate by asymmetrically reducing 4-bromo-3-oxobutanoate, a DNA construct having a promoter in operative linkage

with the polynucleotide sequence, a recombinant vector comprising the polynucleotide sequence, a transformant, a recombinant vector and the like.

EP 1 213 354 A3



European Patent
Office

EUROPEAN SEARCH REPORT

Application Number
EP 01 31 0251

DOCUMENTS CONSIDERED TO BE RELEVANT			
Category	Citation of document with indication, where appropriate, of relevant passages	Relevant to claim	CLASSIFICATION OF THE APPLICATION (Int.Cl.7)
X	EP 0 967 271 A (KANEGAFUCHI CHEMICAL IND) 29 December 1999 (1999-12-29)	1-19	C12N15/53 C12N9/04
Y	* the whole document *	1-23, 30-37	C12N1/21 C12P7/42 C12P7/62
X	EP 1 013 758 A (DAICEL CHEM) 28 June 2000 (2000-06-28) * the whole document *	1-19	
Y	EP 0 606 899 A (DAICEL CHEM) 20 July 1994 (1994-07-20) * the whole document *	1-22	
X	WO 00 71503 A (UNIV MICHIGAN) 30 November 2000 (2000-11-30)	24-29	
Y	* page 10, line 11 - page 11, line 16 *	30-37	
Y,D	WANG J -C ET AL: "Cloning, sequence analysis, and expression in Escherichia coli of the gene encoding phenylacetaldehyde reductase from styrene-assimilating Corynebacterium sp. strain ST-10." APPLIED MICROBIOLOGY AND BIOTECHNOLOGY, vol. 52, no. 3, 1999, pages 386-392, XP002221908 ISSN: 0175-7598 * the whole document *	23	
A	WO 99 42590 A (BIRCH OLWEN ;KIENER ANDREAS (CH); LONZA AG (CH); THOENI SUSANNE (C) 26 August 1999 (1999-08-26) --/--		
The present search report has been drawn up for all claims			
Place of search MUNICH		Date of completion of the search 21 November 2002	Examiner Kania, T
CATEGORY OF CITED DOCUMENTS X : particularly relevant if taken alone Y : particularly relevant if combined with another document of the same category A : technological background O : non-written disclosure P : intermediate document T : theory or principle underlying the invention E : earlier patent document, but published on, or after the filing date D : document cited in the application L : document cited for other reasons A : member of the same patent family, corresponding document			

EPO FORM 1503 (03/02) (P04/00)



European Patent
Office

Application Number

EP 01 31 0251

CLAIMS INCURRING FEES

The present European patent application comprised at the time of filing more than ten claims.

- ☐ Only part of the claims have been paid within the prescribed time limit. The present European search report has been drawn up for the first ten claims and for those claims for which claims fees have been paid, namely claim(s):
- ☐ No claims fees have been paid within the prescribed time limit. The present European search report has been drawn up for the first ten claims.

LACK OF UNITY OF INVENTION

The Search Division considers that the present European patent application does not comply with the requirements of unity of invention and relates to several inventions or groups of inventions, namely:

see sheet 8

- ☒ All further search fees have been paid within the fixed time limit. The present European search report has been drawn up for all claims.
- ☐ As all searchable claims could be searched without effort justifying an additional fee, the Search Division did not invite payment of any additional fee.
- ☐ Only part of the further search fees have been paid within the fixed time limit. The present European search report has been drawn up for those parts of the European patent application which relate to the inventions in respect of which search fees have been paid, namely claims:
- ☐ None of the further search fees have been paid within the fixed time limit. The present European search report has been drawn up for those parts of the European patent application which relate to the invention first mentioned in the claims, namely claims:



European Patent
Office

EUROPEAN SEARCH REPORT

Application Number
EP 01 31 0251

DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document with indication, where appropriate, of relevant passages	Relevant to claim	CLASSIFICATION OF THE APPLICATION (Int.Cl.7)
A	KATAOKA M ET AL: "Stereoselective reduction of ethyl 4-chloro-3-oxobutanoate by Escherichia coli transformant cells coexpressing the aldehyde reductase and glucose dehydrogenase genes." APPLIED MICROBIOLOGY AND BIOTECHNOLOGY, vol. 51, no. 4, April 1999 (1999-04), pages 486-490, XP002209640 ISSN: 0175-7598 ----	20,22	TECHNICAL FIELDS SEARCHED (Int.Cl.7)
A	WO 93 18138 A (KERNFORSCHUNGSANLAGE JUELICH) 16 September 1993 (1993-09-16) ----		
A	DATABASE EMBL 'Online! 12 October 1998 (1998-10-12) KIMURA M.: "Gibberella zeae gene for reductase" retrieved from EBI Database accession no. AC014493; 074646 XP002209648 * 77% identity to SEQ ID NO:2; 73% identity to SEQ ID NO:1 * * abstract * ----		
A	DATABASE WPI Week 198546, 1985 Derwent Publications Ltd., London, GB; AN 1985-287883 XP002209642 & JP 60 199393 A (SUMITOMO CHEM IND KK), 8 October 1985 (1985-10-08) * abstract * ----- -/--		
The present search report has been drawn up for all claims			
Place of search MUNICH		Date of completion of the search 21 November 2002	Examiner Kania, T
CATEGORY OF CITED DOCUMENTS X : particularly relevant if taken alone Y : particularly relevant if combined with another document of the same category A : technological background O : non-written disclosure P : intermediate document T : theory or principle underlying the invention E : earlier patent document, but published on, or after the filing date D : document cited in the application L : document cited for other reasons & : member of the same patent family, corresponding document			



European Patent
Office

LACK OF UNITY OF INVENTION
SHEET B

Application Number

EP 01 31 0251

The Search Division considers that the present European patent application does not comply with the requirements of unity of invention and relates to several inventions or groups of inventions, namely:

1. Claims: 1-22 completely

A polynucleotide according to SEQ ID NO:2 encoding a polypeptide according to SEQ ID NO:1 preferentially producing (S)-4-bromo-3-hydroxybutanoate by asymmetrically reducing 4-bromo-3-oxobutanoate, and related subject-matter. A process for producing (S)-4-halo-3-hydroxybutanoate using said polypeptide.

A process for producing an optically active 3-hydroxybutanoic acid ester employing a microorganism belonging to *Penicillium citrinum*, especially *P. citrinum* IF04631.

A process for producing an optically active 3-hydroxybutanoic acid ester employing a microorganism belonging to *Cryptococcus humicolus*, especially *C. humicolus* IF01527.

A process for producing an optically active 3-hydroxybutanoic acid ester employing a microorganism belonging to *Bacillus alvei*, especially *B. alvei* IF03343t.

1.1. Claims: 1-19 completely; 20-22 partially

A polynucleotide according to SEQ ID NO:2 encoding a polypeptide according to SEQ ID NO:1 preferentially producing (S)-4-bromo-3-hydroxybutanoate by asymmetrically reducing 4-bromo-3-oxobutanoate, and related subject-matter. A process for producing (S)-4-halo-3-hydroxybutanoate using said polypeptide. A process for producing an optically active 3-hydroxybutanoic acid ester employing a microorganism belonging to *Penicillium citrinum*, especially *P. citrinum* IF04631.

1.2. Claims: 20-22 partially

A process for producing an optically active 3-hydroxybutanoic acid ester employing a microorganism belonging to *Cryptococcus humicolus*, especially *C. humicolus* IF01527.

1.3. Claims: 20-22 partially

A process for producing an optically active 3-hydroxybutanoic acid ester employing a microorganism belonging to *Bacillus alvei*, especially *B. alvei* IF03343t.



European Patent
Office

LACK OF UNITY OF INVENTION
SHEET B

Application Number

EP 01 31 0251

The Search Division considers that the present European patent application does not comply with the requirements of unity of invention and relates to several inventions or groups of inventions, namely:

2. Claim : 23 completely

A process for producing an optically active 4-bromo-3-hydroxybutanoate by reacting the substrate with an enzyme having the amino acid sequence according to SEQ ID NO:34, encoded by the polynucleotide of SEQ ID NO:35, and related subject-matter.

3. Claims: 24-37 completely

A process for producing 4-cyano-3-hydroxybutanoic acid or an ester thereof by chemical synthesis, or conducting the asymmetrical reduction reaction by a microorganism belonging to *Penicillium citrinum*, especially *P. citrinum* IF04631, and related subject-matter.

Please note that all inventions mentioned under item 1, although not necessarily linked by a common inventive concept, could be searched without effort justifying an additional fee.



European Patent
Office

EUROPEAN SEARCH REPORT

Application Number
EP 01 31 0251

DOCUMENTS CONSIDERED TO BE RELEVANT			
Category	Citation of document with indication, where appropriate, of relevant passages	Relevant to claim	CLASSIFICATION OF THE APPLICATION (Int.Cl.7)
A	<p>DATABASE CA 'Online! CHEMICAL ABSTRACTS SERVICE, COLUMBUS, OHIO, US; TAKAHASHI, HIDEYUKI ET AL: "Microbial production of D-.alpha.-amino acids" retrieved from STN Database accession no. 107:76090 XP002209641 * abstract * & JP 62 025990 A (KANEKAFUCHI CHEMICAL INDUSTRY CO., LTD., JAPAN) 3 February 1987 (1987-02-03)</p>	20,22	<p>TECHNICAL FIELDS SEARCHED (Int.Cl.7)</p>
A	<p>ITOH NOBUYA ET AL: "Production of chiral alcohols by enantioselective reduction with NADH-dependent phenylacetaldehyde reductase from Corynebacterium strain, ST-10." JOURNAL OF MOLECULAR CATALYSIS B ENZYMATIC, vol. 6, no. 1-2, 4 January 1999 (1999-01-04), pages 41-50, XP002221909 ISSN: 1381-1177 * the whole document *</p>	23	
A	<p>ITOH NOBUYA ET AL: "Purification and characterization of phenylacetaldehyde reductase from a styrene-assimilating Corynebacterium strain, ST-10." APPLIED AND ENVIRONMENTAL MICROBIOLOGY, vol. 63, no. 10, 1997, pages 3783-3788, XP002221910 ISSN: 0099-2240</p> <p style="text-align: center;">-/-</p>	23	
The present search report has been drawn up for all claims			
Place of search MUNICH		Date of completion of the search 21 November 2002	Examiner Kania, T
<p>CATEGORY OF CITED DOCUMENTS</p> <p>X : particularly relevant if taken alone Y : particularly relevant if combined with another document of the same category A : technological background O : non-written disclosure P : intermediate document</p> <p>T : theory or principle underlying the invention E : earlier patent document, but published on, or after the filing date D : document cited in the application L : document cited for other reasons & : member of the same patent family, corresponding document</p>			

EPO FORM 1503 03/02 (P4/C01)



European Patent
Office

EUROPEAN SEARCH REPORT

Application Number
EP 01 31 0251

DOCUMENTS CONSIDERED TO BE RELEVANT			
Category	Citation of document with indication, where appropriate, of relevant passages	Relevant to claim	CLASSIFICATION OF THE APPLICATION (Int.Cl.7)
A	TROOSTWIJK C B ET AL: "METHOD FOR THE SYNTHESIS OF 4-SUBSTITUTED ACETOACETATES" JOURNAL OF THE CHEMICAL SOCIETY, CHEMICAL COMMUNICATIONS, CHEMICAL SOCIETY, LETCHWORTH, GB, no. 23, 7 December 1977 (1977-12-07), pages 932-933, XP000571420 ISSN: 0022-4936 • the whole document *	24-37	
A	US 4 978 768 A (VON DER CRONE JOST ET AL) 12 December 1990 (1990-12-18) • the whole document *	25, 27, 36, 37	
E	EP 1 201 647 A (SUMITOMO CHEMICAL CO) 2 May 2002 (2002-05-02) • the whole document *	23	
T	ITOH NOBUYA ET AL: "Chiral alcohol production by NADH-dependent phenylacetaldehyde reductase coupled with in situ regeneration of NADH." EUROPEAN JOURNAL OF BIOCHEMISTRY, vol. 269, no. 9, May 2002 (2002-05), pages 2394-2402, XP002221911 =ejb&page=aims May, 2002 ISSN: 0014-2956	23	
The present search report has been drawn up for all claims			TECHNICAL FIELDS SEARCHED (Int.Cl.7)
Place of search MUNICH		Date of completion of the search 21 November 2002	Examiner Kania, T
<p>CATEGORY OF CITED DOCUMENTS</p> <p>X : particularly relevant if taken alone Y : particularly relevant if combined with another document of the same category A : technological background O : non-written disclosure P : intermediate document</p> <p>T : theory or principle underlying the invention E : earlier patent document, but published on, or after the filing date D : document cited in the application L : document cited for other reasons S : member of the same patent family, corresponding document</p>			

EPO FORM 1503 03/02 (P04C01)

ANNEX TO THE EUROPEAN SEARCH REPORT
ON EUROPEAN PATENT APPLICATION NO.

EP 01 31 0251

This annex lists the patent family members relating to the patent documents cited in the above-mentioned European search report. The members are as contained in the European Patent Office EDP file on
The European Patent Office is in no way liable for these particulars which are merely given for the purpose of information.

21-11-2002

Patent document cited in search report		Publication date		Patent family member(s)	Publication date
EP 0967271	A	29-12-1999	AU	4032997 A	26-08-1998
			EP	0967271 A1	29-12-1999
			SI	20120 A	30-06-2000
			SK	105699 A3	12-06-2000
			US	6218156 B1	17-04-2001
			WO	9835025 A1	13-08-1998
			US	2002006651 A1	17-01-2002
EP 1013758	A	28-06-2000	JP	2000236883 A	05-09-2000
			EP	1013758 A2	28-06-2000
			US	6312933 B1	06-11-2001
			US	2002042110 A1	11-04-2002
			US	2002127679 A1	12-09-2002
EP 0606899	A	20-07-1994	JP	3155107 B2	09-04-2001
			JP	6209782 A	02-08-1994
			CA	2113240 A1	13-07-1994
			DE	69422064 D1	20-01-2000
			DE	69422064 T2	25-05-2000
			EP	0606899 A2	20-07-1994
			US	5559030 A	24-09-1996
WO 0071503	A	30-11-2000	US	6114566 A	05-09-2000
			AU	4662900 A	12-12-2000
			EP	1180095 A1	20-02-2002
			WO	0071503 A1	30-11-2000
WO 9942590	A	26-08-1999	AU	2926599 A	06-09-1999
			CA	2311649 A1	26-08-1999
			WO	9942590 A1	26-08-1999
			EP	1054974 A1	29-11-2000
			JP	2002504337 T	12-02-2002
WO 9318138	A	16-09-1993	CA	2117482 A1	16-09-1993
			WO	9318138 A1	16-09-1993
			DE	59306681 D1	10-07-1997
			DK	630402 T3	22-12-1997
			EP	0630402 A1	28-12-1994
			JP	7505770 T	29-06-1995
			US	5523223 A	04-06-1996
JP 60199393	A	08-10-1985	JP	1822917 C	10-02-1994
			JP	5032035 B	14-05-1993
JP 62025990	A	03-02-1987	JP	1048758 B	20-10-1989
			JP	1561892 C	31-05-1990

EPO FORM P445B

For more details about this annex : see Official Journal of the European Patent Office, No. 12/82

**ANNEX TO THE EUROPEAN SEARCH REPORT
ON EUROPEAN PATENT APPLICATION NO.**

EP 01 31 0251

This annex lists the patent family members relating to the patent documents cited in the above-mentioned European search report. The members are as contained in the European Patent Office EDP file on. The European Patent Office is in no way liable for these particulars which are merely given for the purpose of information.

21-11-2002

Patent document cited in search report		Publication date		Patent family member(s)	Publication date
US 4978768	A	18-12-1990	CH	664954 A5	15-04-1988
			DE	3700675 A1	16-07-1987
			FR	2601005 A1	08-01-1988
			GB	2185482 A ,B	22-07-1987
			IT	1201152 B	27-01-1989
			JP	1985107 C	25-10-1995
			JP	7023351 B	15-03-1995
			JP	62169755 A	25-07-1987
			KR	9408916 B1	28-09-1994
EP 1201647	A	02-05-2002	EP	1201647 A2	02-05-2002
			JP	2002201169 A	16-07-2002
KR 2000015622	A	15-03-2000	NONE		